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## ERRATA AND AUTHORS' EMENDATIONS

- Page 17, line 2, "*(Saccharum officinarum L.)*" should be "(hybrids of *Saccharum officinarum L.*, *S. barberi* Jes, *S. spontaneum* Jes)."
- Page 41, table 2, column 4, third line from bottom, "9.240" should be "90 240."
- Page 50, line 2, "decreases" should be "increases."
- Page 50, second line from bottom, "decreased" should be "increased "
- Page 83, line 23, "life of many other plants" should be "life of the pollen of many other plants "
- Page 123, tenth line from bottom, omit "apparently."
- Opposite page 235, legend for plate 2, "optically active" should be "anisotropic."
- Page 327, fourth line from bottom, "(p. 324)" should be "(p. 332) "
- Page 341, last line, "330" should be "332 "
- Page 350, seventeenth line from bottom, "from" should be "to "
- Page 497, line 13, "*M.C.±e*," should be "*M.C.±e*", line 14, "*M.C.±e*" should be "*M.C.±e* "
- Page 536, table 1, column 1, "5" should be "45."
- Page 541, footnote 4, volume number given as 11 should be 2.
- Page 596, legend for figure 7, "*mexican*" should be "*mericana*."
- Page 633, line 15, "March 8 to May 22, 1926" should be "March 28 to May 16, 1926 "
- Page 730, fourth line from bottom, after "variety" add "with the exception of hydrogen ions in Valencia rinds as recorded in table 5."
- Pages 732-742, tables 2, 3, 4, and 8, omit "*Percent*" in columns referring to "Flavedo-albedo ratio." Table 8, also omit "*Percent*" in column referring to "Reducing-invert sugar ratio "
- Page 735, table 4, "*Pct.*" under "Hydrogen-ion concentration" should be "*pH*"
- Page 744, last line, after "oranges" add "with the exception of hydrogen ions in Valencia rinds as recorded in table 5 "
- Pages 750-763, tables 1, 2, 3, 4, and 6, omit "*Percent*" in columns referring to "Flavedo-albedo ratio "
- Pages 760 and 781, plates 1 and 2, which should appear opposite pages 760 and 781, respectively, were inadvertently omitted. These illustrations have been sent to all who receive the Journal.
- Page 815, footnote 3, "and" should be "ended."
- Page 826, footnote 2, should be "Collected July 8, hard-dough stage, July 18, ripe stage."
- Page 833, table 12, line 5, "Double Cross 2305" should be "Double Cross 2302."
- Page 875, table 9, right-hand box heading under Spray 7F, "Survival" should be "Corrected survival "
- Page 967, figure 5, the value of the coordinate of the point whose abscissa is 1.4 and whose ordinate is 1.9 should be shown as 1.09.
- Page 968, figure 6, the value of the coordinate of the point whose abscissa is 1.0 and whose ordinate is 1.7 should be shown as 0.76.



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NO. 1

## REACTION OF OAT VARIETIES TO PHYSIOLOGIC RACES OF LOOSE AND COVERED SMUTS OF RED OATS<sup>1</sup>

By GEORGE M. REED, *curator, Brooklyn Botanic Garden*, and T. R. STANTON, *senior agronomist, Division of Cereal Crops and Diseases, Bureau of Plant Industry, United States Department of Agriculture*

### INTRODUCTION

Investigations by Reed (5, 6)<sup>2</sup> and Reed and Stanton (9) demonstrated the existence of races of loose smut, *Ustilago avenae* (Pers.) Jens., and covered smut, *U. levis* (Kell. and Sw.) Magn., that attack Fulghum and related strains of red oats. When the Fulghum oat first came into prominence, a desirable character was its supposed resistance to the smuts. Reed (3) carried out an extensive series of experiments with numerous varieties of oats by the use of inoculum of loose and covered smuts collected in the vicinity of Columbia, Mo. Fulghum was one of the few that stood out prominently as very resistant. These results, by means of still more extensive experiments, were confirmed by Reed, Griffiths, and Briggs (8). Salmon and Parker (10) and Stadler (13) also emphasized the importance of the smut resistance of Fulghum and some of its selections. However, an increasing prevalence of smut in Fulghum oats in the Southern and South Central States has been observed by the junior writer in recent years. There also is more smut in the Red Rustproof strains than formerly. Owing to the extensive culture of the Fulghum type of oats from spring seeding, loose smut is particularly common on that type in Kansas, Oklahoma, and Texas. In the Southeastern States, particularly Georgia and South Carolina, both loose and covered smuts on Fulghum are fairly common.

C. O. Johnston, associate pathologist, Division of Cereal Crops and Diseases, reported from Manhattan, Kans., on the prevalence of Fulghum smut as follows:<sup>3</sup>

Loose smut continues prevalent in Texas, Oklahoma, and Kansas. Most of the smut is the form that is virulent on Fulghum. This form apparently came from the winter-sown oats of the South. With the discovery and rapid adoption of the Fulghum type of oats, this form of smut increased and rapidly moved northward, until it is now coextensive with the distribution of Fulghum. In 1934 occasional fields sown with untreated seed had as much as 25 percent of smut. Covered smut is occasionally found in Kansas and was observed on a few hybrid selections at Stillwater, Okla.

In order to obtain further information on the resistance and susceptibility of oat varieties to these new smut races, the present investigation was undertaken.

<sup>1</sup> Received for publication Sept. 26, 1935; issued February 1936. Cooperative Investigations by the Division of Cereal Crops and Diseases, Bureau of Plant Industry, U. S. Department of Agriculture, and the Brooklyn Botanic Garden. Brooklyn Botanic Garden Contribution no. 72.

<sup>2</sup> Reference is made by number (italics) to Literature Cited, p. 15.

<sup>3</sup> JOHNSTON, C. O. U. S. Dept. Agr., Bur. Plant Indus., Div. Cereal Crops and Diseases Cereal Courier 26: 97. 1934. [Mimeographed]

## PREVIOUS INVESTIGATIONS ON PHYSIOLOGIC SPECIALIZATION OF OAT SMUTS

The discovery by Reed (4), in 1924, of physiologic races of oat smuts lent a new interest to the question of varietal resistance of oats. The first evidence of specialization was obtained by a comparison of loose and covered smuts from Missouri and from Wales, Great Britain. The results of Reed were confirmed by Sampson (11).

As soon as the existence of specialized races in the oat smuts was demonstrated, Reed (5) secured collections of loose smut from red oats grown in the South to determine whether specialization of the smut occurred on the red-oat group of varieties. Reed found that three collections of loose smut on Fulghum, obtained from Tennessee, Texas, and Oklahoma, very severely infected Fulghum but not Red Rustproof. Loose smut obtained on Red Rustproof from Texas infected the Red Rustproof but not the Fulghum type. Thus two distinct races of loose smut on the red oats were demonstrated.

Further data on the races adapted, respectively, to Fulghum and to Red Rustproof, as well as on other specialized races of loose and covered smuts, were published in 1929 by Reed (6). At that time, 11 races of loose smut and 5 races of covered smut were recognized. Sampson (12) published additional data on the occurrence of specialization of the oat smuts in Wales. Nicolaisen (2), in Germany, also has recorded data for many collections of loose smut in which he has found marked differences in reaction on oat varieties.

In 1932 Reed and Stanton (9) published results obtained with additional collections of loose smut on red-oat varieties. Loose smut on Norton from State College, Miss., on Kanota from Newton, Kans., and on Frazier from Lawton, Okla., gave 100-percent infection on Fulghum and high percentages of infection on Canadian and Victor, while negative results were secured with Red Rustproof and Navarro. Three collections of loose smut from Denton, Tex., and from Stillwater and Lawton, Okla., on strains of the Red Rustproof variety severely infected Red Rustproof and also Canadian; negative results were obtained on Navarro, and only a slight smutting of Victor occurred. Fulghum was resistant to two collections, while in the case of the smut from Lawton, Okla., 30 percent of the Fulghum plants were smutted. This, however, probably was due to a mixture of the two races in the original collection of spores.

The occurrence of a specialized race of covered smut that attacks Fulghum oats was recorded in the same paper (9). Two collections of this smut were used, one from Athens, Ga., and the other from Clemson College, S. C. Very high percentages of infection on Fulghum, Canadian, and Victor were secured with both collections, negative results being obtained with Red Rustproof and Navarro.

In 1932 Reed (7) demonstrated that Black Mesdag was also susceptible to the *Ustilago levis* race of Fulghum. This variety previously had manifested an unusual resistance to all hitherto known races of both loose and covered smut, and it has been widely used in oat-smut studies and hybridization experiments in different parts of the world.

## IMPORTANCE OF FULGHUM OATS

During the last 15 years the Fulghum oat has become one of the most important commercial varieties in the United States. It was first reported in 1912 in southeastern Georgia, having been developed

by J. A. Fulghum, of Warrenton, Ga., who observed an exceptional plant in his field, which had been sown with Texas Rustproof. Whether this plant was a mechanical mixture or a hybrid between Red Rustproof and some other variety is not definitely known.

Fulghum is grown as a fall-sown oat in the Southern States and California. It has, however, attained economic importance in Missouri, Kansas, and Oklahoma as a spring-sown variety. Stanton (14), Stanton and Coffman (15, 16), Stanton, Love, and Down (17), Salmon and Parker (10), and Stadler (13) have reported on the characters and economic value of the variety and its selections, including Frazier and Kanota.

## MATERIALS AND METHODS

### COLLECTIONS OF LOOSE AND COVERED SMUTS

The present paper records the data for 5 collections of covered smut and 13 collections of loose smut, obtained for the most part from Fulghum and related oats. These collections were tested on more than 30 oat varieties to determine the range of specialization of the two races of smut. The older and the more recently acquired collections of both loose and covered smuts were used. The history of these collections is shown in table 1.

TABLE 1.—*History of collections of covered smut (Ustilago levis) and loose smut (U. avenae) used in tests on red oats*

Species and collection no.	Host	Collector	Locality	Year
<i>Ustilago levis</i> :				
11.....	Fulghum.....	T. R. Stanton.....	Athens, Ga.....	1929
12.....	do.....	do.....	Clemson College, S. C.....	
17.....	do.....	do.....	Tifton, Ga.....	
18.....	do.....	do.....	do.....	1932
20.....	Black Mesdag.....	T. R. Stanton and V. C. Hubbard.....	Mandan, N. Dak.....	
<i>Ustilago avenae</i> .				
12.....		L. R. Hesler.....	Knoxville, Tenn.....	1924
13.....	Fulghum.....	T. R. Stanton.....	Lawton, Okla.....	1925
14.....	do.....	P. B. Dunkle.....	Denton, Tex.....	
17.....	do.....	G. J. Wilds.....	Hartsville, S. O.....	
18.....	Norton.....	T. R. Stanton.....	State College, Miss.....	1929
19.....	Kanota.....	do.....	Newton, Kans.....	
20.....	Frazier.....	do.....	Lawton, Okla.....	
35.....	Fulghum.....	do.....	Experiment, Ga.....	1932
36.....	do.....	do.....	Tifton, Ga.....	
21.....	Red Rustproof.....	do.....	Denton, Tex.....	
22.....	do.....	do.....	Stillwater, Okla.....	1929
38.....	do.....	do.....	Experiment, Ga.....	
39.....	Nortex.....	do.....	Roslyn, Va.....	

Four of the collections of covered smut were obtained in the Southeastern States by the junior writer. All of them were made from fields sown to Fulghum. *Ustilago levis*-11 and *U. levis*-12 were collected in 1929 and were used in the previous experiments. *U. levis*-17 and *U. levis*-18 were obtained in May 1932.

*Ustilago levis*-20 was collected on Black Mesdag by T. R. Stanton and V. C. Hubbard at the United States Northern Great Plains Field Station, Mandan, N. Dak., in August 1932. The variety was grown in an experimental plot in which the influence of smut infection on plant vigor in oats was being studied (1). The appearance of covered smut on Black Mesdag at Mandan is of exceptional interest. This smut from Mandan also attacked some hitherto resistant hybrid selections, of which Black Mesdag was one of the parents at the Aberdeen Substation, Aberdeen, Idaho, in 1934. These selections

were tested for the department of field crops, University of Alberta, Edmonton, Canada. The original smut used for inoculating Black Mesdag at Mandan in 1932 was a mixture of several collections of spores of *U. levis*, furnished by V. F. Tapke, Bureau of Plant Industry, United States Department of Agriculture. In view of the results of experiments reported herein, the material used evidently contained some smut spores originally collected on Fulghum.

Of the 13 collections of loose smut used in the present experiments (table 1), 9 collections, several of which were used in the earlier experiments, were from the Fulghum type of oats, and 4 were from strains of the Red Rustproof variety.

Some of the collections as originally received were mixtures, and the early tests demonstrated the fact that two or more distinct races were present. The original field of oats from which the smuts were gathered, while predominantly of one variety, evidently contained admixtures of others.

#### OAT SPECIES AND VARIETIES INOCULATED

In the course of all the experiments, 34 varieties or strains of oats, belonging to 6 species and subspecies of *Avena*, were used to differentiate the smut races. Definite strains of these varieties, many of them grown for many years in connection with the various experiments of the senior writer, were used. These particular strains are now recognized as of special significance in the differentiation of specialized races of loose and covered smuts of oats.

The common oat, *Avena sativa* L., was represented by 17 varieties; the common side oat, *A. sativa orientalis* (Schreb.) Alefeld, by 2 varieties; the naked oat, *A. nuda* L., by 2 varieties; and the red oat, *A. byzantina* C. Koch, by 6 varieties. In addition, one or more strains of *A. barbata* Brot., *A. brevis* Roth, *A. fatua* L., *A. strigosa* Schreb., and *A. sterilis* L. were grown.

#### METHODS

All of the experiments were conducted at the Brooklyn Botanic Garden in the period from 1931 to 1934. Some of the smut cultures were grown in the greenhouse, but most of them were grown in the field. The methods employed have been developed by the senior writer in connection with his various experiments with the oat smuts. After removing the hulls, the caryopses were treated for 10 minutes with a solution of formaldehyde, 1 part to 320. They were then inoculated by dusting with the dry spores, and germinated at a temperature of approximately 20° C. in sand with a low moisture content. Under these conditions the seedlings usually emerged in 4 days, and 2 to 3 days later were transplanted. The experiments with most of the varieties were repeated one or more times to insure reliable results.

#### EXPERIMENTAL DATA

##### TESTS WITH FULGHUM COLLECTIONS OF *USTILAGO LEVIS*

On the basis of the reaction to the five smut collections, the oat varieties are arranged in three classes: (1) Varieties susceptible to all collections; (2) varieties resistant to some collections and susceptible to others; and (3) varieties resistant to all collections. Within each of these classes the varieties are further grouped as to species.

The detailed data for the five collections of covered smut are given in table 2.

TABLE 2.—Reaction of species and varieties of *Avena* to collections of *Ustilago levis* on Fulghum and Black Mesdag oats grown at Brooklyn Botanic Garden, 1931-34

## CLASS 1, SUSCEPTIBLE TO ALL COLLECTIONS

Species and variety	Seed no. <sup>1</sup>	C. I. no. <sup>2</sup>	Reaction of <i>Avena</i> plants to collection 3 of—									
			U. levis-11		U. levis-12		U. levis-17		U. levis-18		U. levis-20	
			Inoculated	Smutted	Inoculated	Smutted	Inoculated	Smutted	Inoculated	Smutted	Inoculated	Smutted
<i>Avena byzantina</i> :			Number	Percent	Number	Percent	Number	Percent	Number	Percent	Number	Percent
Fraser.....	914	2381	35	45.7	71	40.8	35	77.1	37	75.6	19	84.2
Fulghum.....	129	3211	117	49.5	119	46.9	53	83.0	58	77.5	36	77.7
Do.....	1000	708	39	61.5	60	43.3	18	100.0	38	86.8	20	95.0
Kanota.....	906	889	78	53.8	85	52.9	50	76.0	57	68.4	20	95.0
<i>Avena nuda</i> :												
Hull-less.....	30	3007	88	14.7	86	66.2	19	84.2	36	58.3	32	81.2
Liberty.....	292	845	17	5.8	14	35.7	20	70.0	17	82.3	18	11.1
<i>Avena sativa</i> :												
Black Diamond.....	116	1878	53	10.3	67	53.7	20	10.0	30	13.3	34	47.0
Black Mesdag.....	70	1877	152	45.3	119	61.3	74	35.1	82	52.4	87	59.7
Canadian.....	119	1625	68	98.5	25	100.0	25	100.0	26	100.0	43	100.0
Early Champion.....	160	1886	90	42.2	58	79.3	18	22.2	30	26.6	30	53.3
Joanette.....	187	1762	75	73.3	73	89.0	18	83.8	51	51.6	35	80.0
Monarch.....	161	1876	110	98.1	112	97.3	49	93.8	52	96.1	52	98.0
Victor.....	126	1875	82	74.3	35	85.7	18	100.0	31	90.3	32	96.8
<i>Avena sativa orientalis</i> :												
Green Mountain.....	110	1892	43	37.5	73	32.8	19	57.8	26	61.5	26	65.3

## CLASS 2, RESISTANT TO SOME COLLECTIONS AND SUSCEPTIBLE TO OTHERS

Species and variety	Seed no.	C. I. no.	Reaction of <i>Avena</i> plants to collection 3 of—									
			U. levis-11		U. levis-12		U. levis-17		U. levis-18		U. levis-20	
			Inoculated	Smutted	Inoculated	Smutted	Inoculated	Smutted	Inoculated	Smutted	Inoculated	Smutted
<i>Avena byzantina</i> :			Number	Percent	Number	Percent	Number	Percent	Number	Percent	Number	Percent
Fraser.....	288	1783	71	0	59	8.4	4	0	9	0	10	0
Fulghum.....	175	1861	17	35.2	14	0	17	23.5	32	6.2	36	22.2
<i>Avena sativa</i> :												
Black Norway.....	118	1874	39	69.2	15	0	30	0	45	0	36	0
Drum Island.....	149	2210	59	1.6	38	2.6	38	0	59	0	39	7.6
<i>Avena stripera</i> :												
<i>Avena sativa</i> , <i>oreocaudata</i> intermedia Marx.....	587	1782	67	0	70	44.2	12	0	32	9.3	15	13.3
	590	3214	18	5.5	15	6.6	7	100.0	10	100.0	14	0

See footnotes at end of table.



TABLE 2.—Reaction of species and varieties of *Avena* to collections of *Ustilago levis* on Fulghum and Black Mesdag oats grown at Brooklyn Botanic Garden, 1931-34—Continued

CLASS 3, RESISTANT TO ALL COLLECTIONS

Species and variety	Seed no. <sup>1</sup>	C. I. no. <sup>2</sup>	Reaction of <i>Avena</i> plants to collection <sup>3</sup> of—									
			<i>U. levis</i> -11		<i>U. levis</i> -12		<i>U. levis</i> -17		<i>U. levis</i> -18		<i>U. levis</i> -20	
			Inoculated	Smutted	Inoculated	Smutted	Inoculated	Smutted	Inoculated	Smutted	Inoculated	Smutted
			Number	Percent	Number	Percent	Number	Percent	Number	Percent	Number	Percent
<i>Avena byzantina</i> :												
Navarro.....	939	966	60	0	36	0	18	0	38	0	16	0
Red Rustproof.....	990	1815	18	0	15	0	16	0	16	0	13	0
<i>Avena fatua</i> .....	968	3213	41	0	34	42.9	15	0	33	0	31	0
<i>Avena sativa</i> :												
Corinthian.....	928	1242	15	0	17	0	15	0	25	0	34	0
Danish.....	369	1609	55	0	37	0	15	0	37	0	36	0
Goldland.....	132	1898	59	0	37	0	38	0	55	0	55	0
Irish.....	690	2329	19	0	12	0	17	0	16	0	16	0
Madison.....	712	2053	50	0	30	0	11	0	27	0	28	0
March Selection.....	192	1683	55	0	37	0	15	0	36	0	33	0
Rossman.....	322	1688	57	0	39	0	19	0	38	0	34	0
Scottish Chief.....	124	1901	56	0	35	0	12	0	33	0	37	0
<i>Avena sativa orientalis</i> :												
Selzire.....	246	1609	56	0	39	0	18	0	34	0	36	0
<i>Avena sterilis, macrocarpa</i> Monsch.....	1002	2657	14	0	12	0	14	0	16	0	35	0

<sup>1</sup> Special numbers assigned to strains of these varieties inoculated and observed by the senior writer.

<sup>2</sup> C. I. refers to accession number of the Division of Cereal Crops and Diseases, formerly Office of Cereal Investigations.

<sup>3</sup> *Ustilago levis*-20, on Black Mesdag, Mandan, N. Dak. Other collections on Fulghum at following localities: *U. levis*-11, Athens, Ga.; *U. levis*-12, Clemson College, S. C.; *U. levis*-17 and -18, Tifton, Ga.

<sup>4</sup> Probably off-type plant or natural hybrid.

## VARIETIES SUSCEPTIBLE TO ALL COLLECTIONS

Four of the collections were made on Fulghum in Georgia, Mississippi, and South Carolina, and therefore special interest is attached to the reaction of Fulghum and related varieties. In addition to two strains of Fulghum, Frazier and Kanota were inoculated. All five collections of covered smut produced smut in all of these varieties. The percentage of smutting on Fulghum (129)<sup>4</sup> ranged from 46.9 percent with *Ustilago levis*-12 to 83 percent with *U. levis*-17, and that on Fulghum (1000) from 43.3 percent with *U. levis*-12 to 100 percent with *U. levis*-17. Frazier gave 40.8 percent infection with *U. levis*-12 to 84.2 percent with *U. levis*-20, and Kanota 52.9 percent with *U. levis*-12 to 95 percent with *U. levis*-20.

Perhaps next in interest to the behavior of these Fulghum varieties is the fact that Black Mesdag proved susceptible to all the collections: the percentage of smutting ranged from 35.1 with *Ustilago levis*-17 to 61.3 percent with *U. levis*-12. As noted earlier, it is probable that the smut collected on Black Mesdag at Mandan, N. Dak., came originally from spores obtained in the South.

Canadian, Joannette, Monarch, and Victor gave rather high percentages of infection with all five collections. These varieties usually are susceptible to specialized races of covered smut. Monarch, in particular, has shown high susceptibility to practically all such races while being very resistant to most races of loose smut.

The varieties Black Diamond, Early Champion, Hull-less, Liberty, and Green Mountain generally gave much lower percentages of smut than the varieties just mentioned. While infections were obtained on these varieties with all the collections, the percentage varied greatly. Whether any significance may be attached to this variability has not been determined.

## VARIETIES RESISTANT TO SOME COLLECTIONS AND SUSCEPTIBLE TO OTHERS

Burt, a red oat, gave negative results with *Ustilago levis*-12 and low percentages of smut with the other four collections. The great variability of Burt in plant characters may be paralleled by a similar variability in its reaction to the races of covered smut.

Black Norway gave negative results with four collections. However, of 39 plants inoculated with *Ustilago levis*-11, 27, or 69.2 percent, were smutted. This result seems definitely to differentiate this collection from the other four.

Danish Island gave negative results with two collections and very slight infections with *Ustilago levis*-11, *U. levis*-12, and *U. levis*-20.

A strain of *Avena brevis* gave negative results with four collections. However, 8.4 percent of the plants were infected with *Ustilago levis*-12. The smutted plants were similar to those occasionally attacked by the Missouri race of covered smut. The plants first sent up normal stems of the usual height. A few tillers infected with smut developed from the base, attaining one-half to two-thirds the height of the normal stalks.

The results with strain no. 587 of *Avena strigosa* probably are significant in separating *Ustilago levis*-12 from the other four collections,

<sup>4</sup> Numbers in parentheses indicate special numbers assigned to strains of these varieties inoculated and observed by George M. Reed.

since 44.2 percent of the plants were infected with *U. levis*-12, whereas negative results were secured with *U. levis*-11 and *U. levis*-17 and very low percentages of smutted plants with *U. levis*-18 and *U. levis*-20.

*Intermedia*, a variety of *Avena strigosa orcadensis* Marq., also has given significant results. All of the plants inoculated with *Ustilago levis*-17 and *U. levis*-18 were smutted. Infection occurred in 5.5 percent of the plants inoculated with *U. levis*-11 and in 6.6 percent of those inoculated with *U. levis*-12. *U. levis*-20 gave negative results. This variety was tested in only one series of experiments and, on account of late seeding, did not fully head, so that the number of plants in a few of the series is rather low for drawing definite conclusions.

#### VARIETIES RESISTANT TO ALL COLLECTIONS

Eight varieties of *Avena sativa*—Cornellian, Danish, Gothland, Iogold, Markton, Monarch Selection, Rossman, and Scottish Chief—and Seizure, a variety of *A. sativa orientalis*, proved to be resistant to all collections. It may be noted that nearly all these varieties are resistant to specialized races of covered smut while very susceptible to many races of loose smut.

Navarro and Red Rustproof, varieties of *Avena byzantina*, as well as *macrocarpa*, a variety of the wild red oat, *A. sterilis*, were resistant to all collections of *Ustilago levis*.

One strain of the common wild oat, *Avena fatua*, was resistant, although one smutted plant inoculated with *Ustilago levis*-12 was noted in the series.

#### TESTS WITH FULGHUM COLLECTIONS OF USTILAGO AVENAE

There were 9 collections of loose smut, 5 of which came from Tennessee, South Carolina, Mississippi, and Georgia, and 4 from Kansas, Oklahoma, and Texas. So far as definitely known, most of the collections were secured on Fulghum, while *Ustilago avenae*-20 was made on Frazier and *U. avenae*-18 on Norton, a variety originally developed from a cross in which Fulghum was one of the parents. The varieties are classified as before, i. e., on the basis of their reaction to the collections of loose smut. The data are recorded in table 3.

#### VARIETIES SUSCEPTIBLE TO ALL COLLECTIONS

Frazier gave a range of infection from 48.2 percent with *Ustilago avenae*-19 to 79.4 percent with *U. avenae*-18. Fulghum (129) ranged from 54.5 percent with *U. avenae*-35 to 74.3 percent with *U. avenae*-20. Fulghum (1000) ranged from 25 percent infection with *U. avenae*-35 to 95 percent with *U. avenae*-18. Kanota gave a range of 47.3 percent with *U. avenae*-35 to 70.5 percent with *U. avenae*-14. In no case were all the inoculated plants of these varieties infected.

Canadian gave high percentages of smutted plants with all of the collections. Somewhat variable results were obtained with Black Diamond, Victor, Hull-less, Liberty, and Green Mountain. Rather high percentages of infection usually were secured with most of the collections.

The strain of *Avena barbata* proved susceptible to six of the collections. Unfortunately, the number of plants was small because the seed was sown late and very poor heading resulted; although the variety was used with the other collections, no fully developed plants were secured.

TABLE 3.—Reaction of species and varieties of *Avena* to collections of *Ustilago avenae* on Frazier, Fulghum, Kanola, and Norton oats grown at the Brooklyn Botanic Garden, 1931-34

## CLASS 1, SUSCEPTIBLE TO ALL COLLECTIONS

Species and variety	Seed no.	C. I. no.	Reaction of <i>Avena</i> plants to collection 1 of—											
			<i>U. avenae</i> -12			<i>U. avenae</i> -13			<i>U. avenae</i> -14			<i>U. avenae</i> -17		
			Inocu- lated	Num- ber	Per- cent	Inocu- lated	Num- ber	Per- cent	Inocu- lated	Num- ber	Per- cent	Inocu- lated	Num- ber	Per- cent
<i>Avena barbata</i> .....	830	2467	8	37.5	37.5	6	66.6	66.6	16	100.0	100.0	8	79.4	79.4
<i>Avena byzantina</i> .....	914	2381	56	55.3	55.3	53	64.1	64.1	56	69.6	69.6	53	52.8	52.8
Frazier.....	129	3211	76	55.2	74	66.2	74	66.2	76	73.6	73.6	87	63.2	61.0
Fulghum.....	1000	708	39	69.2	39	87.1	37	81.0	37	81.0	71.7	20	95.0	40
Do.....	906	839	50	64.0	54	61.6	51	70.5	51	70.5	49.0	36	61.1	53
Kanola.....	30	3007	16	56.2	17	66.6	17	66.6	20	80.0	80.0	53	86.7	38
<i>Avena nuda</i> .....	202	845	17	94.1	17	41.1	17	41.1	19	78.9	78.9	20	100.0	19
Full-less.....	116	1878	18	16.6	19	78.9	18	78.9	18	22.2	22.2	15	53.3	37
<i>Avena sativa</i> .....	119	1625	96	100.0	48	95.8	56	100.0	56	100.0	32	100.0	71	91.5
Black Diamond.....	126	1875	57	50.8	54	57.4	54	57.4	53	13.2	13.2	78	42.3	76
Victor.....	110	1892	18	61.1	18	83.3	17	83.3	17	35.2	35.2	20	75.0	39
<i>Avena sativa orientalis</i> .....														
Green Mountain.....														

## CLASS 2, RESISTANT TO SOME COLLECTIONS AND SUSCEPTIBLE TO OTHERS

<i>Avena byzantina</i> .....	175	1861	34	14.7	30	13.3	34	11.7	34	29.4	35	22.8	35	5.7	33	3.0	32	9.3	32	15.6
Burt.....	968	3213	19	36.8	16	0	15	0	18	0	46	0	29	0	17	0	18	0	18	0
<i>Avena sativa</i> .....	118	1874	17	17.6	19	21.0	18	5.5	19	21.0	37	27.0	15	6.6	19	0	18	0	18	0
Black Norway.....	309	1669	20	5.0	17	41.1	19	52.6	19	5.2	58	1.7	37	37.8	18	11.1	18	0	18	0
Danish.....	149	3210	20	0	18	0	19	84.2	17	5.8	50	8.4	39	17.9	19	0	20	0	17	5.8
Danish Island.....	150	1886	20	60.0	18	72.2	19	68.4	19	52.6	57	21.0	50	30.0	17	0	18	0	20	27.7
Early Champion.....	152	1898	36	0	37	21.6	38	41.6	40	0	76	0	59	25.4	37	45.9	39	0	40	0
Gotthard.....	161	1876	36	0	36	0	38	0	40	7.5	76	1.4	58	0	37	67.2	39	0	40	0
Monarch.....	161	1876	36	0	36	0	38	0	40	7.5	76	1.4	58	0	37	67.2	39	0	40	0
Monarch Selection.....	162	1879	70	0	68	82.3	73	41.0	73	0	90	0	91	61.5	68	16.2	68	19.6	54	23.0
Rossmann.....	322	1888	19	0	15	6.6	17	41.1	18	0	57	0	40	32.5	20	15.0	20	0	20	0
Scottish Chief.....	124	1901	17	0	19	5.2	19	5.2	19	21.0	57	3.5	36	0	18	0	19	0	20	1.8
<i>Avena sativa orientalis</i> .....																				
Seizure.....	246	1609	17	0	17	58.8	19	42.1	18	0	53	0	39	23.0	18	27.7	19	0	19	0
<i>Avena strigosa orcadensis</i> .....																				
<i>Avena infernalis</i> .....	590	3214	15	0	15	0	18	0	18	0	17	82.3	16	0	15	0	18	0	15	0

See footnotes at end of table.

TABLE 3.—Reaction of species and varieties of *Avena* to collections of *Ustilago avenae* on Frazier, Fulghum, Kanola, and Norton oats grown at the Brooklyn Botanic Garden, 1931-34—Continued

CLASS 3, RESISTANT TO ALL COLLECTIONS

Species and variety	Seed no.	C. I. no.	Reaction of <i>Avena</i> plants to collection 1 of—											
			<i>U. avenae</i> -12		<i>U. avenae</i> -13		<i>U. avenae</i> -14		<i>U. avenae</i> -17		<i>U. avenae</i> -18		<i>U. avenae</i> -19	
			Inoculated	Smuted	Inoculated	Smuted	Inoculated	Smuted	Inoculated	Smuted	Inoculated	Smuted	Inoculated	Smuted
<i>Avena brevis</i> .....	289	1783	Num-ber 14	Per-cent 0	Num-ber 11	Per-cent 0	Num-ber 15	Per-cent 0	Num-ber 46	Per-cent 0	Num-ber 31	Per-cent 0	Num-ber 18	Per-cent 0
<i>Avena byzantina</i> .....	939	966	16	0	18	0	20	0	59	0	39	0	20	0
<i>Avena sativa</i> .....	999	1815	39	0	39	0	18	0	19	0	37	0	38	0
<i>Avena sativa</i> .....	70	1877	18	0	18	0	16	0	57	0	35	0	18	0
Black Maslag.....	928	1242	16	0	18	0	17	0	19	0	18	0	20	0
Cornelian.....	930	2329	18	0	18	0	20	0	18	0	20	0	19	0
Jogold.....	187	1762	18	0	17	0	19	0	37	0	40	0	19	0
Joanette.....	752	2053	19	0	19	0	20	0	32	0	37	0	18	0
Markton.....	1002	2657	12	0	16	0	12	0	13	0	14	0	17	0
<i>Avena sterilis macrocarpa</i> .....	587	1782	20	0	17	0	20	0	38	0	38	0	19	0
<i>Avena striosa</i> .....														

1 *U. avenae*-12, host variety unknown, Knoxville, Tenn.; *U. avenae*-13, on Norton, State College, Miss.; *U. avenae*-19, on Kanola, Newton, Kans.; *U. avenae*-20, on Frazier, Lawton, Okla.; others on Fulghum at following localities: *U. avenae*-14, Denton, Tex.; *U. avenae*-17, Hartsville, S. C.; *U. avenae*-35, Experiment, Ga.; *U. avenae*-36, Fulton, Ga.

\* Infection probably due to off-type plants or natural hybridization.

## VARIETIES RESISTANT TO SOME COLLECTIONS AND SUSCEPTIBLE TO OTHERS

Black Norway, Monarch, and Scottish Chief (varieties of *Avena sativa*) and Burt (a variety of *A. byzantina*) gave rather variable results. The percentage of smutting usually was very low and sometimes negative. Whether any special significance may be attached to the results obtained with these varieties has not been determined.

However, the results with some other varieties indicate differences in the specialization of the collections. *Ustilago avenae*-12, *U. avenae*-17, *U. avenae*-18, *U. avenae*-35, and *U. avenae*-36, which were collected in Tennessee, Georgia, Mississippi, and South Carolina, can be separated from *U. avenae*-13, *U. avenae*-14, *U. avenae*-19, and *U. avenae*-20, collected in Kansas, Oklahoma, and Texas, by the reaction of Gothland, Monarch Selection, Seizure, and Rossman. *U. avenae*-13, *U. avenae*-14, *U. avenae*-19, and *U. avenae*-20 have given comparatively high percentages of infection on all these varieties, while low percentages or negative results have been secured with the remaining collections. The results perhaps are most striking with Monarch Selection, a large number of plants having been inoculated with the various collections of smuts. The results were entirely negative with *U. avenae*-12, *U. avenae*-17, and *U. avenae*-18, while with *U. avenae*-35, 19.6 percent of the plants, and with *U. avenae*-36, 1.8 percent were smutted. On the other hand, *U. avenae*-13 gave 82.3 percent; *U. avenae*-14, 41 percent; *U. avenae*-19, 61.5 percent; and *U. avenae*-20, 67.6 percent.

It is probable that the results with Danish Island may serve to differentiate collection *Ustilago avenae*-14, since 84.2 percent of infection was obtained. With the other collections, the results were negative or gave comparatively low percentages of infection.

It is also possible that the strain of *Avena fatua* may separate *Ustilago avenae*-12 from the other collections, since 36.8 percent of the plants were smutted, while negative results were obtained in all the other series.

*Intermedia*, a variety of *Avena strigosa orcadensis*, seems to separate *Ustilago avenae*-18 from the other collections, since 82.3 percent of the inoculated plants were smutted, while negative results were obtained with all the other collections.

## VARIETIES RESISTANT TO ALL COLLECTIONS

Navarro and Red Rustproof, varieties of *Avena byzantina*, gave negative results with all of the collections, although one smutted plant of Red Rustproof, inoculated with *Ustilago avenae*-13, was noted. *Macrocarpa*, a variety of *A. sterilis*, gave negative results with the eight collections with which it was inoculated.

Black Mesdag, Cornellian, Iogold, Joannette, and Markton gave negative results, although smutted plants of Cornellian inoculated with *Ustilago avenae*-14 and *U. avenae*-36 and one smutted plant of Markton inoculated with *U. avenae*-19 were noted.

The strain of *Avena brevis* gave negative results with eight of the collections, no plants maturing in the series with *Ustilago avenae*-13. Strain no. 587 of *A. strigosa* also gave completely negative results.

TESTS WITH RED RUSTPROOF COLLECTIONS OF *USTILAGO AVENAE*

Previous investigations by Reed (5, 6) and Reed and Stanton (9) demonstrated the existence of a specialized race of loose smut that

attacks Red Rustproof and closely related oat varieties. Additional data on four collections of loose smut on Red Rustproof and Nortex have been obtained. In the course of the experiments, 27 strains or varieties belonging to various species of *Avena* have been inoculated. Nearly all of these have been grown in the three series in which the smut was obtained on Red Rustproof, while seven varieties were inoculated with the smut obtained on Nortex. The data are given in table 4.

TABLE 4.—Reaction of species and varieties of *Avena* to collections of *Ustilago avenae* on Red Rustproof oats grown at the Brooklyn Botanic Garden, 1931-34

Species and variety	Seed no.	C. I. no.	Reaction of <i>Avena</i> plants to collection <sup>1</sup> of—							
			<i>U. avenae</i> -21		<i>U. avenae</i> -22		<i>U. avenae</i> -38		<i>U. avenae</i> -39	
			Inoculated	Smuted	Inoculated	Smuted	Inoculated	Smuted	Inoculated	Smuted
			Num-ber	Per-cent	Num-ber	Per-cent	Num-ber	Per-cent	Num-ber	Per-cent
<i>Avena byzantina</i> :										
Fulghum.....	129	3211	40	0	20	0	20	0	-----	-----
Navarro.....	939	966	38	0	11	0	18	0	-----	-----
Nortex.....	907	2342	-----	-----	11	90.9	20	20.0	20	55.0
Red Rustproof.....	131	3212	17	35.2	20	25.0	-----	-----	-----	-----
Do.....	265	884	20	85.0	20	50.0	18	27.7	-----	-----
Do.....	999	1815	-----	-----	20	35.0	20	40.0	20	75.0
<i>Avena brevis</i> .....	289	1783	37	0	20	0	-----	-----	-----	-----
<i>Avena fatua</i> .....	968	3213	32	96.8	25	100.0	36	77.7	19	100.0
<i>Avena strigosa</i> .....	587	1782	35	57.1	35	37.1	12	0	-----	-----
<i>Avena nuda</i> :										
Hull-less.....	30	3007	39	0	19	0	19	0	-----	-----
<i>Avena sativa</i> :										
Black Diamond.....	116	1878	38	2.6	16	0	19	0	-----	-----
Black Mesdag.....	70	1877	38	0	19	0	18	0	-----	-----
Canadian.....	119	1825	39	61.5	40	57.5	37	48.6	16	81.2
Danish.....	309	1669	40	0	20	0	18	0	-----	-----
Danish Island.....	149	3210	37	2.7	20	0	19	0	-----	-----
Early Champion.....	150	1886	36	5.5	20	5.0	18	0	-----	-----
Gothland.....	152	1898	34	0	19	0	38	0	20	0
Joanette.....	187	1762	39	0	20	0	19	0	-----	-----
Markton.....	752	2053	39	0	20	0	18	0	-----	-----
Monarch.....	161	1876	39	0	20	0	17	0	-----	-----
Monarch Selection.....	162	1879	39	0	19	0	17	0	-----	-----
Rossmann.....	322	1688	39	0	20	0	19	0	-----	-----
Scottish Chief.....	124	1901	33	0	19	0	20	0	-----	-----
Victor.....	126	1875	38	2.6	20	10.0	39	0	19	5.2
<i>Avena sativa orientalis</i> :										
Green Mountain.....	110	1892	38	0	20	0	19	0	-----	-----
Seizure.....	246	1909	36	0	20	0	18	0	-----	-----
<i>Avena sterilis macrocarpa</i> .....	1002	2657	-----	-----	-----	-----	18	0	19	15.7

<sup>1</sup> *U. avenae*-39 was obtained on Nortex at Rosslyn, Va. Other collections were obtained on Red Rustproof at the following localities. *U. avenae*-21, Denton, Tex.; *U. avenae*-22, Stillwater, Okla.; *U. avenae*-38, Experiment, Ga.

Strains of four varieties of *Avena byzantina* were inoculated with most of the collections. The three strains of Red Rustproof were infected by every collection with which they were inoculated. Nortex, a similar variety, also was infected with the three collections of smut with which it was inoculated. Fulghum and Navarro gave negative results.

Canadian was the only variety of *Avena sativa* severely infected, the percentages ranging from 48.6 to 81.2. The varieties Black Mesdag, Danish, Gothland, Joanette, Markton, Monarch, Monarch Selection, Rossmann, and Scottish Chief gave negative results. An occasional smuted plant of Black Diamond, Danish Island, Early Champion, and Victor was observed.

Green Mountain and Seizure (varieties of *Avena sativa orientalis*), Hull-less (a variety of *A. nuda*), and the strain of *A. brevis* also gave negative results. *Macrocarpa*, a variety of *A. sterilis*, was grown in only two of the series, and in one of them three smuted plants were observed. Strain no. 587 of *A. strigosa* gave 57.1 percent infection

with *Ustilago avenae*-21 and 37.1 percent with *U. avenae*-22, while negative results were obtained with *U. avenae*-38. However, only 12 plants were grown in the experiments with the last collection.

The results obtained with *Avena fatua* are especially interesting. One strain was inoculated with all four collections, and the percentages of infection ranged from 77.7 to 100. It is evident that this strain of the species is very susceptible to the race of loose smut that attacks strains of the Red Rustproof variety.

#### DISCUSSION

The data recorded confirm the results of earlier investigations, indicating clearly that there are distinct races of loose and covered smuts specialized to the Fulghum type of red oats. These races, however, are not restricted to these oats but may attack varieties belonging to other species and subspecies of *Avena*.

Four collections of covered smut used in these experiments were obtained on Fulghum in Georgia and South Carolina, and one collection was obtained on Black Mesdag at Mandan, N. Dak. The evidence indicates, however, that the spores that infected Black Mesdag were introduced from the South.

Specialization within the races of *Ustilago levis*-Fulghum and *U. avenae*-Fulghum is also indicated. *U. levis*-11 is differentiated from the other collections by its ability to infect Black Norway, a variety unusually resistant to the races of covered smut. In the present experiments, 39 plants were grown and 27 (69.2 percent) were smutted. The results with strain no. 587 of *A. strigosa* probably separate *U. levis*-12 from the other four collections. Significant results with *intermedia*, a variety of *A. strigosa oreadenis*, also have been obtained. This variety has proved to be very susceptible to *U. levis*-17 and *U. levis*-18 and resistant to the other collections.

Further specialization also is indicated in the collections of loose smut. Gothland, Monarch Selection, Seizure, and Rossman appear to be susceptible to *Ustilago avenae*-13, *U. avenae*-14, *U. avenae*-19, and *U. avenae*-20, while practically negative results have been secured with the remaining collections. It is also possible that Danish Island may serve to differentiate *U. avenae*-14 from the other collections, since 84.2 percent of the plants were smutted.

The strain of *Avena fatua* was infected with only one collection of *Ustilago avenae*-Fulghum, the plants inoculated with *U. avenae*-12 giving 36.8 percent infection.

The Fulghum races of covered and loose smuts have shown very distinct differences in their reaction on the various oat varieties. Both groups severely attack the strains of Fulghum and are negative on such varieties as Navarro and Red Rustproof. Both loose and covered smuts infect severely some varieties of the *Avena sativa* group, for example, Canadian. Other varieties, however, are completely resistant to the loose smut but susceptible to the covered smut. It is especially interesting that Black Mesdag is resistant to the loose smut but susceptible to the covered smut.

The race of loose smut collected from strains of the Red Rustproof variety appears to be more limited in its capacity for infecting oat varieties. It is confined to strains of the Red Rustproof type belonging to *Avena byzantina*. Fulghum and Navarro are completely



resistant. Only one variety of the common oat, Canadian, is seriously attacked. Sporadic infections seem to occur on some other varieties, including Black Diamond, Danish Island, Early Champion, and Victor. Black Mesdag and Markton are among the varieties conspicuous for their resistance. The varieties of *A. nuda* and *A. sativa orientalis* also proved resistant. The strain of *A. brevis* was entirely free from smut. The one strain of *A. strigosa* grown contained a considerable number of smutted plants. It is especially interesting that the specific strain of *A. fatua* inoculated was fully susceptible to this race. This strain was collected in California in 1930.

#### SUMMARY

Five collections of *Ustilago levis* have been tested on 34 strains or varieties of oats belonging to species of *Avena*. Four collections were obtained on Fulghum in Georgia and South Carolina, and one on Black Mesdag in North Dakota. These collections proved to be highly specialized in their ability to attack certain varieties of oats. They were very similar in behavior, but minor differences, indicating the existence of subraces, were evident.

Nine collections of *Ustilago avenae*, from the southern oat-growing region, were used for inoculation experiments. Five of the collections came from Tennessee, Georgia, Mississippi, and South Carolina, and four from Kansas, Oklahoma, and Texas. These collections were similar in their reaction on many oat varieties, but some evidence was obtained indicating that specialized subraces may occur.

A distinct race of loose smut occurs on Red Rustproof oats. Canadian (a variety of *Avena sativa*) and the strains of *A. fatua* and *A. strigosa* grown in the present experiments are also susceptible.

It is noteworthy that Markton and Navarro stand out as completely resistant to all these collections of loose and covered smuts. Black Mesdag, hitherto recognized as extremely resistant to all races of smuts, was found to be decidedly susceptible to *Ustilago levis*-Fulghum, although completely resistant to *U. avenae*-Fulghum and *U. avenae*-Red Rustproof.

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# CHEMICAL COMPOSITION OF JUICE FROM LOUISIANA SUGARCANE INJURED BY THE SUGARCANE BORER AND THE RED ROT DISEASE<sup>1</sup>

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## INTRODUCTION

In a study of the effect of fertilizers and soil conditions on the composition of the juice of Louisiana sugarcane (*Saccharum officinarum* L.), unusual variations in the purity and in the nonsugars of the juice of samples were occasionally found. The fact that these samples of irregular composition appeared to be largely confined to varieties known to be highly susceptible to damage by the sugarcane borer (*Diatraea saccharalis* F.) and red rot (*Colletotrichum falcatum* Went) led to the belief that such injury had adversely affected the composition of the juice. Recently, Hinds et al. (4)<sup>3</sup> have shown that borer infestation materially reduces the purity of cane juice. They have thus confirmed the earlier work of Cross, Allbright, Van Dine, Barber, Stubbs, Morgan, and others, which has been summarized by Holloway (5), and which clearly indicates that both the quality and the quantity of the juice are reduced and the reducing sugars in the juice are increased by injury from borer infestation. In the original description of the red rot disease in Java, Went (10) presented juice analyses showing that even a light infection of the stalks by the causal fungus resulted in a significant decrease of sucrose and a corresponding increase of invert sugars. This result has also been demonstrated by Edgerton (3) in Louisiana. Little or no information seems available on the effect of injury to sugarcane by borers or red rot on the various nonsugars of the juice, the nonsugars being the salts and organic compounds other than sucrose and reducing sugars. Therefore, analyses of the juice of sound and of injured canes have been made to determine whether borer and red rot injury had the same effect on the nonsugars as on the carbohydrate fraction of the juice. As a result of these investigations, reported herein, previous observations on the effect of borer and red rot injury on extraction and juice purity are confirmed, and further data have been obtained which indicate that these injuries materially alter the quantity and composition of the nonsugars in the juice.

## MATERIALS AND METHODS

On November 27, 1931, during the harvest of certain experimental plots of plant sugarcane grown on Sharkey silty clay at the United States Sugar Plant Field Station near Houma, La., a number of

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<sup>3</sup> Reference is made by number (italic) to Literature Cited, p. 24.

apparently equally mature mill-length stalks of the four varieties shown in table 1 were collected, split lengthwise, and examined for borer and red rot injury. From this material three samples of each variety were selected: (1) Wholly sound stalks, (2) stalks with an approximately equal amount of borer injury but free from red rot, and (3) stalks showing approximately uniform amounts of both red rot and borer damage. Each sample thus obtained consisted of about 10 stalks of cane. The percentages of infestation were recorded at the time of collection of the samples and are expressed as percentages of internodes affected. Immediately after collection the samples were crushed in a hydraulically equipped sugarcane mill and the juice freed of bagassillo, sediment, and foam by a standardized procedure developed by the writers. The resulting "standard raw crusher juice" was analyzed for juice solids with Brix spindles and for apparent sucrose by direct polarization of the undiluted juice after clarification with dry basic lead acetate (9). Acidity was determined by titration of 10 cc of juice, after dilution with distilled water, with 0.1 N alkali, using phenolphthalein as indicator. The total nitrogen was determined by the Gunning method (2), protein nitrogen by precipitation with tannic acid, and analysis of the precipitate for nitrogen according to the micro-Kjeldahl method of Pregl (7). Gums were estimated by acid hydrolysis of the alcohol-insoluble precipitate formed by mixing 1 volume of juice with 7 volumes of 95-percent ethyl alcohol and determination of the liberated reducing sugars. The ash was determined by evaporation of a known quantity of juice and subsequent ignition of the sirup in an electric muffle at a temperature below visible redness. With the exception of chloride, which was estimated on a separate portion of juice by a modification of Pettinger's method (6), the individual mineral constituents of the ash were determined by analysis of the ash solution after removal of silica.

In addition to the raw-juice analyses, the juice from the series of samples from the two varieties P. O. J. 213 and P. O. J. 36-M was given a standardized sugar-house clarification. The sulphitation process of clarification was employed, with 0.6 g of sulphur dioxide per liter of juice, the juice being limed cold to such a degree that the clarified juice, after being brought to a boil and settling, had a pH of 6.8 to 7.0. Evaporation of the clarified juice to an effect sirup density of 55° Brix was conducted in a Pyrex glass vacuum pan. These sirups were then analyzed, and thus the corresponding composition of the samples at the stage at which they would be used for boiling to sugar was obtained. The analyses included the determination of true purity (by the Clerget method), reducing sugars, ash, alcohol precipitate, gums, total nitrogen, and the four ash constituents silica, lime, sulphur, and iron. Color and turbidity were estimated by means of a Keuffel & Esser color analyzer. The observed percentage transmission at 560 $\mu$  was calculated to  $-\log t$  values.

On October 31, 1932, a second set of samples of P. O. J. 213 plant cane was collected from a different location on the same soil type at the field station, and on November 3, samples of Co. 281 plant cane were collected from an adjacent plot. These canes differed from those collected in 1931 in that they consisted of the lower 10 joints only. The samples of sound cane showed no evidence of borer

or of red rot; the diseased stalks were extensively damaged by the borer and every joint showed the characteristic red coloration of the red rot fungus. The milling and analytical procedures used on the raw juices were the same as in 1931, except that the acidity was determined by electrometric titration to pH 7.0.

These samples were also clarified by a factory method, this time lime alone being used to yield a clarified juice of pH 6.8 to 7.0. The clarified juice was not boiled to a sirup, but was analyzed for true purity, reducing sugars, ash, and turbidity.

## EXPERIMENTAL RESULTS

### TESTS IN 1931

The data of table 1 fully confirm previous work and show that borer infestation alone reduces the soluble solids, sucrose, and apparent purity of crusher juice below those of comparable sound cane and that the juice extraction is decreased as a result of the injury. The decreased sucrose and purity values indicate losses of available 96° sugar, ranging from 7.9 pounds to over 31 pounds per ton of cane. These values were calculated by the use of the Winter-Carp formula on the assumption of uniform extraction and reduction factors comparable to those obtained commercially (8); if the values obtained were corrected for the decreased extraction incident to the injury, the apparent losses would be greater. When borer damage is accompanied by red rot, the observations indicate that still further reduction in quality of juice may be expected. As further shown in table 1, the combination of borer and red rot injury, to the extent shown, reduces the solids and purity of the juice to values lower than if borer infestation alone were present, except in the case of P. O. J. 36-M. The indicated sugar losses ranged from 40.7 to over 71 pounds of sugar per ton of cane when not corrected for the extraction differences indicated in the table.

TABLE 1.—*Effect of borer and red rot injury on the quantity and quality of crusher juice from 4 varieties of sugarcane, United States Sugar Plant Field Station, Houma, La., 1931*

Variety	Inter-nodes injured by borer	Inter-nodes injured by red rot	Brix	Apparent sucrose	Apparent purity	Indicated 96° sugar per ton of cane		Cane for 1 ton of sugar	Crusher juice extraction
						Total	Loss due to injury		
	Percent	Percent	Degrees	Percent	Percent	Pounds	Pounds	Tons	Percent
P. O. J. 36-M-----	0.0	0.0	16.66	14.09	84.57	191.8	-----	10.43	69.3
	24.2	.0	15.41	12.22	79.30	180.2	31.6	12.48	68.6
	47.5	32.5	13.86	10.24	73.48	128.4	63.4	15.58	67.4
	.0	.0	16.96	14.58	85.97	200.1	-----	10.00	71.6
P. O. J. 213-----	33.3	.0	15.76	13.19	83.69	178.4	21.7	11.21	67.8
	51.7	40.8	12.39	9.82	79.26	128.7	71.4	15.54	68.2
	.0	.0	18.06	15.85	87.76	219.8	-----	9.10	71.1
P. O. J. 234-----	25.8	.0	17.53	15.32	87.39	211.9	7.9	9.44	68.7
	47.5	34.6	16.11	13.33	82.74	179.1	40.7	11.17	67.7
	.0	.0	16.78	14.38	85.70	197.1	-----	10.15	71.1
Co. 281-----	25.0	.0	16.13	13.66	84.70	185.8	11.3	10.76	69.7
	47.5	34.6	14.73	11.67	79.23	152.8	44.3	13.09	68.0

The diseased and sound samples of P. O. J. 213 and P. O. J. 36-M were analyzed further, to determine whether the nonsugars were affected by borer and red rot injury. From the data of table 2 it is seen that the nonsugars were affected as greatly as was the carbohydrate fraction of the juice. No certain division between the changes produced by the borer and that by the fungus can be made from the data since no samples were available that were free from borer injury yet infected with red rot.

TABLE 2.—Composition of nonsugars of crusher juice of sound and of injured<sup>1</sup> sugarcane, expressed as percentage of Brax solids

Variety and condition of cane	Total nitrogen	Protein nitrogen	Nonprotein nitrogen	Gums	Ash	Potassium as K <sub>2</sub> O	Calcium as CaO
P. O. J. 213 <sup>2</sup>	Percent	Percent	Percent	Percent	Percent	Percent	Percent
Wholly sound.....	0.185	0.074	0.091	0.133	2.093	1.075	0.059
Injured by borer.....	.192	.092	.100	.177	3.078	1.634	.069
Injured by borer and red rot.....	.317	.131	.186	.383	5.105	3.034	.092
P. O. J. 36-M:							
Wholly sound.....	.119	.068	.051	.177	2.251	1.019	.072
Injured by borer.....	.154	.079	.075	.196	2.672	1.397	.073
Injured by borer and red rot.....	.206	.100	.106	.246	3.489	1.780	.101
	Magnesium as MgO	Aluminum as Al <sub>2</sub> O <sub>3</sub>	Iron as Fe <sub>2</sub> O <sub>3</sub>	Phosphate as P <sub>2</sub> O <sub>5</sub>	Sulphur as SO <sub>3</sub>	Silica as SiO <sub>2</sub>	Acidity <sup>2</sup>
P. O. J. 213 <sup>2</sup>	Percent	Percent	Percent	Percent	Percent	Percent	Cc
Wholly sound.....	0.110	0.028	0.020	0.332	0.271	0.110	1.35
Injured by borer.....	.110	.044	.045	.334	.350	.116	1.55
Injured by borer and red rot.....	.186	.174	.047	.494	.552	.157	2.05
P. O. J. 36-M:							
Wholly sound.....	.140	.092	.038	.392	.315	.113	1.50
Injured by borer.....	.128	.107	.013	.454	.247	.127	1.90
Injured by borer and red rot.....	.158	.087	.032	.509	.290	.156	2.00

<sup>1</sup> For extent of injury see second and third columns of table 1.

<sup>2</sup> Phenolphthalein titration, cubic centimeters of 0.1 N alkali required to neutralize 10 cc of juice.

The total nitrogen of P. O. J. 213 increased 16 percent and that of P. O. J. 36-M increased 29 percent as a result of borer injury. When accompanied by red rot, the total nitrogen of these samples increased 92 and 73 percent, respectively, above that found for sound cane. Although the protein-nitrogen content of the juice is materially increased as a result of the infestations, the proportion of the total nitrogen in the protein form appears to be somewhat decreased, with a corresponding increase in the nonprotein fraction. The protein nitrogen decreased from 57 percent of the total in the sound P. O. J. 36-M to 51 percent in the borer-injured sample and to 49 percent in the sample injured by both borer and red rot. The data on the 1931 samples of P. O. J. 213 are less conclusive, the protein nitrogen increasing from 45 percent of the total in sound cane to 48 percent in the bored sample, and decreasing to 41 percent in the bored and red rot sample. The 1932 data, however, for P. O. J. 213, show a more definite decrease in the proportion of protein nitrogen to total nitrogen in the juice from damaged cane as compared with that from sound cane.

The acidity of the juice of both varieties was increased by the borer and particularly by the combined action of borer and red rot. The amount of gums present in the juice was also much increased by the

borer and red rot, and, to a lesser extent, by borer damage alone. While borer damage alone increased the ash content of the P. O. J. 213 samples approximately 50 percent, borer damage combined with red rot increased the ash content 144 percent in the samples examined. Similar but somewhat less marked effects were noted in the case of P. O. J. 36-M, where the juice ash was increased 19 percent by borer injury alone and 55 percent by borer injury and red rot combined. The quantity of each inorganic component present in the juice as found in the ash was likewise increased by borer and red rot injury.

The analyses of the sirups produced from these juices are given in table 3.

TABLE 3.—*Composition of sirups of sound and of diseased<sup>1</sup> cane expressed as percentages of true solids*

Variety and condition of cane	True purity	Reducing sugars	Ash	Total non-sugars	Organic non-sugars	Nitrogen as N	Gums	Organic substances precipitated by alcohol
P. O. J. 213	<i>Percent</i>	<i>Percent</i>	<i>Percent</i>	<i>Percent</i>	<i>Percent</i>	<i>Percent</i>	<i>Percent</i>	<i>Percent</i>
Wholly sound.....	91.02	2.67	2.265	6.31	4.05	0.105	0.094	0.60
Injured by borer.....	89.73	2.70	3.163	7.57	4.41	.135	.147	.95
Injured by borer and red rot.....	82.63	5.78	5.364	11.60	6.24	.215	.331	2.33
P. O. J. 36-M:								
Wholly sound.....	89.88	3.66	2.155	6.46	4.30	.074	.092	.76
Injured by borer.....	86.06	6.68	2.776	7.26	4.48	.091	.114	.96
Injured by borer and red rot.....	82.13	9.13	3.645	8.74	5.09	.129	.211	1.17
	Silica as SiO <sub>2</sub>	Sulphur as SO <sub>3</sub>	Iron as Fe <sub>2</sub> O <sub>3</sub>	Calcium as CaO	Increase in CaO due to clarification	Color number <sup>2</sup>	Turbidity number <sup>2</sup>	
P. O. J. 213:	<i>Percent</i>	<i>Percent</i>	<i>Percent</i>	<i>Percent</i>	<i>Percent</i>			
Wholly sound.....	0.061	0.334	0.0012	0.181	0.122	88	88	
Injured by borer.....	.110	.416	.0042	.191	.122	187	386	
Injured by borer and red rot.....	.121	.617	.0056	.437	.345	342	432	
P. O. J. 36-M:								
Wholly sound.....	.078	.393	.0014	.194	.122	56	49	
Injured by borer.....	.064	.438	.0021	.223	.150	103	77	
Injured by borer and red rot.....	.087	.540	.0032	.344	.243	117	280	

<sup>1</sup> For extent of injury, see second and third columns of table 1.

<sup>2</sup> Color and turbidity numbers are 100 times—log *I*. The larger numbers represent greater color and turbidity.

The data show that changes effected by borer and red rot on the juice purity, ash, gums, and nitrogen were carried through to the sirup. In addition, an increased amount of reducing sugars and of organic nonsugars was found in the sirups from injured cane; the color was much darker and the turbidity greater. The increased color and turbidity are especially objectionable because of their detrimental influence on the quality of the sugar. It is to be noted also that the increase in lime content due to clarification is greater in the case of sirup from the cane having red rot. This unusual increase in soluble lime salts cannot be explained solely on the basis of the higher acidity of the juice but may be due to the kind of acids present.

When the values of the various inorganic substances present in the juice are recalculated to percentage composition of the ash, as in table 4, it is seen that the composition of the ash is not greatly altered



as a result of the infection, even though the total quantity is increased. The principal change was an increase in the proportion of potassium in the ash and a decrease in most of the other constituents.

TABLE 4.—*Composition of the ash of crusher juice of sound and of diseased sugarcane*<sup>1</sup>

Variety and condition of cane	Potassium as K <sub>2</sub> O	Calcium as CaO	Magnesium as MgO	Aluminum as Al <sub>2</sub> O <sub>3</sub>	Iron as Fe <sub>2</sub> O <sub>3</sub>	Phosphorus as P <sub>2</sub> O <sub>5</sub>	Sulfur as SO <sub>3</sub>	Silica as SiO <sub>2</sub>
P. O. J. 213	<i>Percent</i>	<i>Percent</i>	<i>Percent</i>	<i>Percent</i>	<i>Percent</i>	<i>Percent</i>	<i>Percent</i>	<i>Percent</i>
Wholly sound.....	51.36	2.82	5.26	1.34	0.96	15.86	12.95	5.26
Injured by borer.....	53.09	2.24	3.57	1.43	1.46	10.85	11.37	3.77
Injured by borer and red rot.....	59.43	1.80	3.64	3.41	.92	9.08	10.81	3.08
P. O. J. 36-M								
Wholly sound.....	45.27	3.20	6.22	4.09	1.69	17.41	13.99	5.02
Injured by borer.....	52.28	2.73	4.79	4.00	.49	16.99	9.24	4.75
Injured by borer and red rot.....	51.02	2.89	4.53	2.49	.92	14.59	8.51	4.47

<sup>1</sup> For extent of injury see second and third columns of table 1.

#### TESTS IN 1932

The 1931 results were confirmed by the 1932 tests on sound and on diseased cane of the varieties P. O. J. 213 and Co. 281. The samples collected in 1932 were intended to represent maximum conditions of natural infection in the case of two varieties commercially grown in Louisiana that have different degrees of susceptibility to these injuries, P. O. J. 213 being highly susceptible to damage by both borer and red rot (1), and Co. 281 being quite resistant to red rot although somewhat susceptible to damage in the field by the borer. As indicated by the analyses given in table 5, the sample of diseased P. O. J. 213 has only 54 percent of the apparent sucrose and 68 percent of the solids content of the juice of comparable healthy cane, thus indicating a loss of over 117 pounds of 96° sugar per ton of cane. The data for Co. 281 show this variety to be less seriously changed in composition than P. O. J. 213, even when apparently 100-percent infected. The soluble solids and apparent sucrose decrease of 13 percent and 20 percent, respectively (table 5), indicate a loss of sugar, due to the diseased condition of over 45 pounds per ton of cane.

TABLE 5.—*Effect of borer and red rot injury on the sucrose content of crusher juice from two varieties of sugarcane, United States Sugar Plant Field Station, Houma, La., 1932*

Variety and condition of cane	Brix	Apparent sucrose	Apparent purity	Indicated 96° sugar per ton of cane		Cane for 1 ton of sugar
				Total	Loss due to injury	
P. O. J. 213	<i>Degrees</i>	<i>Percent</i>	<i>Percent</i>	<i>Pounds</i>	<i>Pounds</i>	<i>Tons</i>
Sound.....	17.61	15.76	90.01	221.3	-----	9.04
Diseased.....	11.96	8.52	71.24	104.2	117.1	19.19
Co. 281						
Sound.....	17.09	14.14	82.74	190.0	-----	10.53
Diseased.....	14.84	11.30	76.15	144.4	45.6	13.85

The nonsugar analyses (table 6), give further evidence that red rot and borer injury have a greater effect on P. O. J. 213 than on Co. 281. The total nitrogen content of the juice from the former increased

125 percent as a result of infection, and that of the latter increased 51 percent. In both varieties the relative proportion of protein and nonprotein nitrogen was changed by the infestation, the protein nitrogen being 67 percent of the total in the juice of sound P. O. J. 213 and 55 percent of the total in the juice of the diseased samples and 69 and 66 percent, respectively, in the sound and diseased Co. 281. The ash content of both varieties increased because of the injury, the increase being over 200 percent in P. O. J. 213 and 37 percent in Co. 281. Nearly all the mineral constituents of the juice appear to be correspondingly increased. Calculation of the ash constituents as percentages of the ash (table 7) confirmed the previous data in that the potassium content of the juice was increased somewhat more than the other components as a result of injury by borer and red rot.

TABLE 6.—*Composition of the nonsugars of crusher juice of sound and of diseased sugarcane, expressed as percentages of Brix solids*

Variety and condition of cane	Total nitrogen	Protein nitrogen	Nonprotein nitrogen	Ash	Potassium as K <sub>2</sub> O	Sodium as Na <sub>2</sub> O	Calcium as CaO
P O J. 213:	<i>Percent</i>	<i>Percent</i>	<i>Percent</i>	<i>Percent</i>	<i>Percent</i>	<i>Percent</i>	<i>Percent</i>
Sound.....	0.060	0.040	0.020	1.673	0.915	0.033	0.029
Diseased.....	.135	.074	.061	5.043	2.865	.071	.070
Co. 281:							
Sound.....	.070	.048	.022	3.191	1.896	.044	.107
Diseased.....	.106	.070	.036	4.376	2.686	.067	.106
	Magnesium as MgO	Manganese as MnO	Phosphorus as P <sub>2</sub> O <sub>5</sub>	Sulphur as SO <sub>3</sub>	Chloride as Cl	Silica as SiO <sub>2</sub>	Acidity <sup>1</sup>
P O J. 213:	<i>Percent</i>	<i>Percent</i>	<i>Percent</i>	<i>Percent</i>	<i>Percent</i>	<i>Percent</i>	<i>Cc</i>
Sound.....	0.064	0.0013	0.409	0.091	-----	0.088	1.22
Diseased.....	.121	.0029	.991	.290	-----	.112	1.74
Co. 281:							
Sound.....	.145	.0020	.441	.275	0.141	.081	2.32
Diseased.....	.138	.0019	.518	.353	.251	.097	2.42

<sup>1</sup> Electrometric titration to pH 7.0, cubic centimeters of 0.1 N alkali required to neutralize 10 cc of juice

TABLE 7.—*Composition of the ash of crusher juice of sound and of diseased sugarcane*

Variety and condition of cane	Potassium as K <sub>2</sub> O	Sodium as Na <sub>2</sub> O	Calcium as CaO	Magnesium as MgO	Manganese as MnO	Phosphorus as P <sub>2</sub> O <sub>5</sub>	Sulphur as SO <sub>3</sub>	Chlorine as Cl	Silica as SiO <sub>2</sub>
P O J. 213	<i>Percent</i>	<i>Percent</i>	<i>Percent</i>	<i>Percent</i>	<i>Percent</i>	<i>Percent</i>	<i>Percent</i>	<i>Percent</i>	<i>Percent</i>
Sound.....	54.69	1.97	1.73	3.83	0.077	24.45	5.44	-----	5.26
Diseased.....	56.81	1.41	1.39	2.40	.058	19.65	5.75	-----	2.22
Co. 281:									
Sound.....	59.42	1.38	3.35	4.54	.063	13.82	8.62	4.42	2.53
Diseased.....	61.38	1.53	2.42	3.15	.043	11.84	8.07	5.74	2.22

The analyses of the clarified juices (table 8) reaffirmed the purity drop in diseased cane, and, as in the previous tests, showed an increase in the proportion of reducing sugars, ash, and organic nonsugars and a poorer degree of clarity given by a standard sugar-house clarification. As in the case of the raw juice, the changes were greater in the clarified juice of P. O. J. 213 than in that of Co. 281.

TABLE 8.—*Composition of lime-clarified juices from sound and from diseased sugarcane, expressed as percentages of true solids*

Variety and condition of cane	True purity	Reduc- ing sugars	Ash	Total non- sugars	Organic non- sugars	Tur- bidity number <sup>1</sup>
P. O. J. 213:	<i>Percent</i>	<i>Percent</i>	<i>Percent</i>	<i>Percent</i>	<i>Percent</i>	
Sound.....	93.87	1.87	1.70	4.26	2.56	43
Diseased.....	80.40	9.06	4.85	10.54	5.69	59
Co. 281						
Sound.....	90.10	3.77	3.00	6.13	3.00	28
Diseased.....	85.00	5.92	4.41	9.18	4.41	40

<sup>1</sup> The turbidity numbers are 100 times the reciprocal of the Kopke value. The larger numbers represent greater turbidity.

## SUMMARY

The composition of juice from sugarcane damaged by the sugarcane borer alone and from cane damaged by both the borer and red rot is contrasted with that of juice from sound cane. The comparisons show that the value of the cane is materially reduced by the borer alone and to a greater extent by the combination of borer and red rot injuries. In both cases the following changes occur as a result of the injuries.

There is a decrease in juice extraction and in the percentage of solids and sucrose in the juice obtained, and the apparent and the true purity are correspondingly lowered.

The percentages of reducing sugars, ash, gums, alcohol-precipitable nonsugars, and total organic nonsugars are significantly increased.

Both protein and nonprotein nitrogen compounds are increased, especially the latter, resulting in a greater increase in the nitrogen content of the sirups.

In the ash, potassium is increased somewhat more than the other elements, although all the mineral constituents of the juice are increased. When calculated to percentage of ash, the ratios between the different components are but slightly altered.

The color and the turbidity of the clarified juices and sirups are decidedly increased.

A comparison of four varieties commercially grown in Louisiana indicates that, in general, the changes in the chemical composition of the juice are greater in the case of the highly susceptible variety P. O. J. 213 than in the other varieties tested.

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# INHERITANCE OF SOME MAJOR COLOR TYPES IN BEETS<sup>1</sup>

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## INTRODUCTION

The occurrence and distribution of red and yellow pigments in beets (*Beta vulgaris* L.), as has generally been recognized, give rise to a large number of heritable color types. But even with such possibilities for genetic study, inheritance in beets has received comparatively little attention, although plant-breeding efforts specially concerned with the sugar content of beets had been in progress for almost a century before the rediscovery of Mendel's principles.

This lack of genetic advancement may in part be attributed to certain characteristics of the beet. Owing to its strong tendency toward self-sterility, populations large enough for study are not readily obtained by inbreeding.<sup>3</sup> When a cross is desired there is usually some selfing. Because of the small size of the beet flower, extensive emasculation is impracticable. Another important reason for the lack of genetic advancement with beets is that many of the important beet characters are readily modified by changes in environmental conditions. In this respect color is no exception. In these studies it was found that the development of both red and yellow root pigments is greatly influenced by the physiological condition of the plant, and under some conditions the effect upon the yellow pigment has been such as to cause serious difficulty in classifying certain genetic phenotypes. A more comprehensive knowledge of the physiological conditions which cause these variations and the discovery of clear-cut genetic characters that can be identified under all conditions will constitute important advances in breeding work with beets.

Kajanus<sup>4</sup> made numerous crosses, which were carried several generations, with beets of the mangel-wurzel type, chiefly of the Eckendorfer and Barres varieties. His data did not lead to a satisfactory genetic analysis because of a persistent and high percentage of contamination, perhaps the result of his use of cloth tents as plant isolators. He was able to present a factorial arrangement, partly hypothetical, by considering large discrepancies to be the effect of contamination. This arrangement assumed a yellow root to be the effect of a dominant factor for yellow, designated *G* (from *Gelb*), and a red root to be the effect of a dominant factor for red, *R*, in

<sup>1</sup> Received for publication Oct. 2, 1935; issued February 1936. Cooperative studies by the U. S. Department of Agriculture and the Utah Agricultural Experiment Station.

<sup>2</sup> The writer acknowledges many helpful suggestions in the preparation of the manuscript from F. V. Owen, geneticist, and other members of the Division of Sugar Plant Investigations, as well as from George Stewart, senior forest ecologist, Intermountain Forest and Range Experiment Station, Forest Service, U. S. Department of Agriculture.

<sup>3</sup> Fully self-fertile strains are now well known to investigators in the Division of Sugar Plant Investigations, but they were not utilized in the study reported in this paper.

<sup>4</sup> KAJANUS, B. ÜBER DIE FARBENVARIATION DER BETA-WÜBEN. Ztschr. Pflanzenzücht. 5:367-372. 1917.

combination with the factor for yellow. A beet of the phenotype *Rg* had a white root, but true whites were of the genotype *rrgg*. He considered these two factors to be independently inherited.

Lindhard and Iversen<sup>5</sup> repeated the work of Kajanus and reached similar conclusions. They believed that some of their data suggested a loose linkage between the *Rr* and *Gg* factor pairs, but this was not consistent with other related data. Rasmuson<sup>6</sup> reported the result of a cross of red beet with a white beet; the color under consideration was presumably confined to the epidermis of the root. Rasmuson thought his data conformed to Kajanus' scheme. Vilmorin<sup>7</sup> studied numerous progenies of "accidental" crosses and reported that the ratios obtained were in general agreement with the findings of Lindhard and Iversen.

The present paper deals with nine color types, obtained from crosses between sugar beets and red beets, and the genetic factors responsible for their inheritance. Crosses were obtained by exposing the mother plant to pollen from the selected male parent. This has been effectively accomplished in two ways: (1) By distance isolation and (2) by the use of paper bags. According to the first method plants were usually grown in nail kegs, and shortly before the flowers opened the paired parent plants were moved to some place at least 200 to 300 yards from the nearest flowering beets. When paper bags were used small branches were bagged shortly before the earliest flowers opened, and a few days later, when about half the flowers had opened and shed their pollen, each bag with its contents was exchanged for a similar bag on the reciprocal parent.

All of the crosses reported in the section on factors *R* and *Y* were made by distance isolation, while most of the crosses in the section on the factors *R'* and *Y'* were made under paper bags. A small amount of contamination may have occurred with either method, but this should not be more serious than in the case of maize or any other cross-pollinated plant.

Both distance isolation and the use of paper bags provide efficient methods of obtaining crosses, but offer no protection against a small percentage of selfing which commonly occurs in beets. It is, of course, important that the breeder be able to recognize those individuals resulting from selfing, and where the parental phenotypes are otherwise similar a color character is frequently extremely useful in distinguishing parentage. If, however, the phenotype of the female parent is expected in the progeny, specific individuals resulting from selfing could not be detected, but the effect of selfing on the population might be recognized by an excessive number of plants in the particular class whose phenotype is also that of the female parent. In the work reported here, the effect of self-fertility has not been entirely avoided, but through the use of simple methods it is believed that pollination has been controlled well enough to permit effective interpretation of the genetic behavior.

<sup>5</sup> LINDHARD, E., and IVERSEN, K. VERERBUNG VON ROTEN UND GELBEN FARBENMERKMALEN BEI BETA-RÜBEN. Ztschr. Pflanzenzücht. 7: 1-18. 1919.

<sup>6</sup> RASMUSON, H. ZUR FRAGE VON DER ENTSTEHUNGSWEISE DER ROTEN ZUCKERRÜBEN. Bot. Notiser 1919: 169-180, illus. 1919.

<sup>7</sup> VILMORIN, J. L. DE. L'HÉRÉDITÉ CHEZ LA BETTERAVE CULTIVÉE. 153 pp., illus. Paris. 1923.

## DESCRIPTION OF COLOR TYPES

At least three color types (red, yellow, and white root) were noted in the literature reviewed, but because of a lack of adequate description of the plants studied it is impossible to establish an accurate relationship between these color types and those which form the basis of the present paper. This paper includes nine color types, which for the purpose of description are divided into two groups: (1) Those containing red pigment and (2) those lacking red pigment.

## TYPES CONTAINING RED PIGMENT

(I) *Red-hypocotyl white root (Ry)*.—The hypocotyl of the seedlings of this type is rose or pale red. The color shows also at the bases of stems and petioles, the intensity depending upon environmental conditions and the stage and rapidity of growth. Genetic modifying factors also may be responsible for different shades and intensities of pigments observed or there may be an allelomorphic series of color factors, each factor having a slightly different expression. Sometimes the pigment is not readily seen in a large vegetative beet, and this may account for some of the confusion in the literature. By cutting down to the central bud, however, as has been pointed out by Nuckols,<sup>8</sup> the pigment can be detected easily. This color character is a specific marker that is expressed at all times, although under certain conditions during the formation of the seedstalk only traces of the pigment remain. Sometimes the pigment also gives part of the skin on the shoulders of the root a tinge of red.

(II) *Red beet (RY)*.—In the cotyledon or seedling stage, the hypocotyl of beets belonging to the red beet type is of an intensely dark or purplish-red color. In the growing and in the mature plant the petiole color may range from a dark purplish red to an orange-red, or it may be red in the middle and orange at the margins. In the leaves the pigment is most concentrated in the midrib and principal veins,<sup>9</sup> but under certain favorable conditions the entire leaf may be dark red. The epidermis of the root is dark red, and the flesh of the root is predominantly red.

(III) *Pale red beet (RY±?)*.—The color in this type extends into every part of the plant characterized by red color in type II, but with greatly reduced intensity.

(IV) *Striped red beet (R'Y)*.—The root and flesh color is intermediate in intensity between types II and III. The color of the petioles is a dark or purplish red similar to that of type II, but it is confined chiefly to stripes. On the upper surface of the petiole the color is usually confined to one broad stripe, but on the underside there are several narrow stripes of red. Orange has never been found on petioles of this type. Only rarely does the red pigment extend into the leaf veins. When it does so, it is extremely dilute.

(V) *Green top red root (RY')*.—The roots of this type vary in color from medium to bright red and cannot be distinguished from the roots of type IV, while in the tops the pigment is generally as restricted as in those of type I. In mature plants there is an abrupt change from the presence of red to its absence or only a trace, at the intersection of crown and petioles. Only one beet of this description was found in 1932, and it became the progenitor of all the beets with green tops and red roots discussed in this paper. The type has probably been observed by earlier workers, but, so far as the writer is aware, there has been no previous description.

(VI) *Red top white root (R'y)*.—Roots of this type are similar to those of type I. They generally show a trace of rose on the shoulders, but under most conditions they are white. The tops are striped with red and cannot be distinguished from those of type IV. In mature plants there is an abrupt change from the presence of intense red to its absence or to a rose tinge, at the intersection of crown and petioles.

## TYPES LACKING RED PIGMENT

(VII) *Yellow hypocotyl white root (ry)*.—In the cotyledon and seedling stages, beets of this type have a whitish-green hypocotyl. Upon exposure to sunlight,

<sup>8</sup> NUCKOLS, S. B. SEEDLING COLOR AND YIELD OF SUGAR BEETS. Jour. Amer. Soc. Agron. 23: 740-743. 1931.

<sup>9</sup> The concentration of pigment in the veins is particularly interesting in comparison with a color type which F. R. Immer obtained from Germany. In the latter type the expression is just opposite, i. e., the pigment in the leaf blades is most concentrated away from the veins.



considerable yellow pigment may develop, however. The bases of the leaves sometimes show a trace of yellow, depending upon environmental conditions, stage of growth, etc. This type corresponds to type I, except that here the pigment is yellow instead of red, and therefore is usually not so conspicuous.

(VIII) *Green top yellow root* (*Yr*).—The petioles and leaves of this type are similar in color to those of type VII, but the epidermis of the root and sometimes part of the flesh and vascular rings are a lemon yellow.

(IX) *Yellow beet* (*rY*).—The yellow pigment in this type is distributed throughout the plant as is the red pigment in type II. The petioles are usually a distinct yellow. The color may be confined to the petioles of young leaves or it may extend into the veins of every green leaf on the plant. When strongly displayed this petiole coloration is a clear golden yellow. The epidermis of the root is lemon yellow except on the shoulders, where in some plants it blends into orange. When the orange is absent the lemon-yellow root color is similar to that of type VIII but is usually more intense. Under some conditions it is impossible to distinguish the roots of this type from those of type VIII.

#### METHOD OF DISTINGUISHING THE YELLOWS OF TYPES VII, VIII, AND IX

In the greenhouse, high temperature and possibly other conditions favorable to rapid growth are so unfavorable for the expression of yellow color as to make it impossible to identify the different types that lack red pigment. Observations made by F. V. Owen have shown that there is a remarkable development of all pigments in the root if young plants grown under greenhouse conditions are pulled and laid in sunlight on damp soil for a few days. Following this exposure to sunlight the presence or absence of factors for yellow pigment can usually be determined. Before the plants were subjected to this treatment, it was particularly difficult to distinguish between the white roots of type VII and the yellow roots of type VIII. Under field conditions, the lemon-yellow color of type VIII is clear-cut and can be easily identified.

#### THE FACTORS R AND Y IN THE RED GARDEN BEET

##### MATERIAL

A beet with a red hypocotyl and a white root, of genetic constitution *Ryry*, was crossed with a red beet of genetic constitution *RYRY*. The cross was made in 1930 by growing the two parent beets side by side in a city garden, where, so far as could be ascertained, they were isolated from other flowering beets by a distance of at least 300 yards. The red-hypocotyl white-root parent (type I) was selected from the P-19 strain<sup>10</sup> (a curly-top resistant strain of sugar beet), and the red beet parent (type II) was a table beet of the Detroit Dark Red variety, a commercial variety characterized by a maximum development of intense red pigment. From this cross, approximately 30 plants were grown from seed harvested from the red-hypocotyl white-root parent (*Ryry*). These  $F_1$  plants were forced to seed under lights in the greenhouse and allowed to cross with each other at random. In 1931 a large population of both the  $F_1$  and  $F_2$  was grown in the field.

If crossing had been complete, all offspring in the  $F_1$  planting would be expected to resemble the dominant red beet parent. This did not prove to be the case, however, and some self-fertilization must have taken place since a few plants with white roots were represented in the progeny. Furthermore, these white-root selfed plants were of two types, the red hypocotyl (*Ry*) and the yellow hypocotyl (*ry*),

<sup>10</sup> Obtained by the Utah Agricultural Experiment Station from Katherine Esau, formerly with the Spreckels Sugar Co., and now a member of the staff of the University of California, Davis, Calif.

which demonstrated that the white-root parent (P-19) was heterozygous for hypocotyl color, and hence of genetic constitution *Ryry*.

Since the composite  $F_2$  obtained could not yield a satisfactory factorial analysis (because of the mixed population of genotypes *RYRy* and *RYry* in the  $F_1$ ) several  $F_1$  plants were backcrossed to the double recessive yellow-hypocotyl white-root type (*ryry*). These crosses, together with further evidence from additional combinations, provided ample proof of the presence of two factors, namely, *Y* for the extension of pigment and *R* for the conversion of yellow to red pigment, which is in line with the results of earlier investigators.

#### $F_1$ BACKCROSSES

The first generation genotypes *RYry* and *RYRy* were both backcrossed to the double recessive *ryry*. Backcrosses of the first group (*RYry*) to the yellow-hypocotyl white-root type (*ryry*) gave four phenotypes, *RY*, *Ry*, *rY*, *ry*, with a close linkage between *R* and *Y*. The data suggest approximately 7.5 percent crossing over. These backcrosses are summarized in table 1, crosses 1 and 2. In each of these crosses several progenies were combined to simplify the presentation. Each progeny had previously been found to be in satisfactory agreement with expectation.<sup>11</sup> The results of these backcrosses are taken as confirming the genetic interpretation given, large deviations from the expected numbers usually being attributed to selfing. Throughout the paper this effect of selfing will be seen operative in accentuating the percentage crossing over or in reducing the value of *P* when the  $\chi^2$  test was used. The interpretations as given have been based upon reasonably consistent behavior of many progenies and are believed to warrant the factorial explanations given.

TABLE 1.—Linkage between the *Rr* and *Yy* factor pairs

Cross no.	Cross	Total number of plants	Number of plants of indicated phenotype				Percentage cross-over $\pm$ P. E.
			Red beet (type II or III) <sup>1</sup>	Red hypocotyl white root (type I)	Yellow beet (type IX)	Yellow hypocotyl white root (type VII)	
			<i>RY</i>	<i>Ry</i>	<i>rY</i>	<i>ry</i>	
1.....	<i>RYry</i> $\times$ <i>ryry</i> .....	484	242	19	18	205	7.6 $\pm$ 0.81
2.....	Reciprocal.....	443	202	20	14	207	7.7 $\pm$ .85
3.....	<i>RYry</i> $\times$ <i>ryry</i> .....	111	58	3	2	48	4.5 $\pm$ 1.33
4.....	Reciprocal.....	654	282	14	14	344	4.3 $\pm$ .53
5.....	<i>RYry</i> $\times$ <i>ryry</i> .....	102	51	2	3	46	4.9 $\pm$ 1.44
6.....	Reciprocal.....	353	172	12	6	163	5.1 $\pm$ .79
7.....	<i>RYry</i> $\times$ <i>RYry</i> .....	67	48	4	1	14	6.3 $\pm$ 2.08
8.....	Reciprocal.....	311	216	10	12	73	7.1 $\pm$ 1.03
9.....	<i>Ryry</i> $\times$ <i>ryry</i> .....	63	4	27	29	3	11.1 $\pm$ 2.88
10.....	Reciprocal.....	41	2	20	12	7	22.0 $\pm$ 4.36
11.....	<i>Ryry</i> $\times$ <i>ryry</i> .....	101	4	50	40	2	5.9 $\pm$ 1.58
12.....	Reciprocal.....	85	2	30	36	17	22.4 $\pm$ 3.05

<sup>1</sup> Type III in crosses 9 to 12.

<sup>11</sup> Probable errors were calculated by the aid of tables prepared by Immer.<sup>12</sup> In some cases the  $\chi^2$  test was most convenient.

<sup>12</sup> IMMER, F. R. FORMULAE AND TABLES FOR CALCULATING LINKAGE INTENSITIES. *Genetics* 15:81-98, illus. 1930.

In backcrosses of the second group ( $RYRy \times rryy$ ), two phenotypes ( $RY$  and  $Ry$ ) were expected in about equal numbers. The actual ratio was 341 to 284, with  $P$  (from  $\chi^2$ ) falling between 0.02 and 0.05; the excess of red plants, however, suggests that there was some self-fertility in the female parent. The reciprocal gave 202  $RY$  to 168  $Ry$ , with  $P$  falling between 0.05 and 0.10. This progeny also contained 24 plants of the yellow-hypocotyl white-root type ( $rryy$ ), but these were without doubt the result of selfing. The figures presented above for the backcrosses of  $RYRy$  to  $rryy$  and the reciprocals are also the sums of several progenies. For the individual progenies, the lowest value for  $P$  was between 0.05 and 0.10 and the highest was between 0.8 and 0.9.

#### F<sub>2</sub> BACKCROSS AND F<sub>3</sub> POPULATIONS

Except that the linkage seems to be slightly closer, the F<sub>2</sub> backcross and F<sub>3</sub> populations are similar to the F<sub>1</sub> backcross populations (crosses 1 and 2) and help to corroborate the genetic interpretation. These data are presented in table 1, crosses 3 to 8, inclusive. In at least one population (cross 4) the closeness of the apparent linkage was accentuated by self-fertility in the yellow-hypocotyl white-root parent ( $rryy$ ).

Additional interest centered in these progenies because in each group one of the red parents showed distinct orange margins on the petioles, while the other red parent had intense red petioles with no evidence of orange. A genetic interpretation for orange margins, as contrasted with lack of orange (both are of color type II), was not obtained because none of the segregates showed a distinct orange, although a few showed a trace. Since the plants were grown in a dense stand, it is possible that orange color requires a particular environment, failing to develop except when the plants are exposed to intense sunshine and there is adequate spacing between plants.

The yellow beet  $rY$  (type IX) was shown to be produced as a simple dominant to the double recessive in a backcross of a heterozygous yellow beet ( $rYry$ ) to the yellow-hypocotyl white-root type ( $rryy$ ). The expected ratio was 1 yellow beet to 1 yellow hypocotyl white root, and the observed numbers were 231 to 253, with  $P$  falling at 0.3.

#### THE PALE RED BEET

The F<sub>1</sub> population grown in 1931 included one unusual plant which was a pale-colored red beet. This plant proved to be self-sterile, but when it was backcrossed to the yellow-hypocotyl white-root type ( $rryy$ ) a total progeny of 290 individuals was obtained. The data are presented in table 1, crosses 9 to 12, inclusive.

Although the linkage could not be accurately measured, chiefly because of self-fertility in the recessive female parents, the ratios (though not the color) were satisfactorily explained by assuming the pale red beet (type III) to be of the genotype  $RyrY$ . This repulsion arrangement is particularly significant because all of the other doubly heterozygous F<sub>1</sub> plants were of the coupling type ( $RYry$ ). However, half of the gametes produced by the sugar beet parent ( $Ryry$ ) were of the  $Ry$  type, and consequently both the repulsion arrangement and the dilute color of the pale red beet could be obtained by the introduction of a gamete of the  $rY^a$  type,  $Y^a$  being a dilute allelomorph of  $Y$ .

There is not sufficient evidence available at present, however, to propose such a factor; consequently, the well-known factor *Y* will be used, it being understood that the correct genotype of the pale red beet has not been determined.

The pale red beet was most likely the result of contamination, but there are other explanations based upon genetic behavior which cannot be entirely disregarded.

#### RECOVERY OF THE PARENTAL TYPES

The parental types *RY* (red beet) and *ry* (yellow hypocotyl white root) have been recovered by crossing the heterozygous recombination types, red hypocotyl white root (*Ryry*)  $\times$  yellow beet (*rYry*). Such a cross constitutes a critical test of the dependability of the genetic interpretation used to explain the color relations observed. The expected ratio 1:1:1:1 is approached by the observed ratios, as shown in table 2. The population obtained from the red-hypocotyl white-root plant as female parent suggests that considerable self-fertilization occurred.

TABLE 2.—A heterozygous yellow beet (*rYry*)  $\times$  a heterozygous red hypocotyl white root (*Ryry*), and the reciprocal (*Ryry*  $\times$  *rYry*)

Cross	Total number of plants	Number of plants of indicated phenotype				P from $\chi^2$ test
		Red beet (type II) <i>RY</i>	Red hypocotyl white root (type I) <i>Ry</i>	Yellow beet (type IX) <i>rY</i>	Yellow hypocotyl white root (type VII) <i>ry</i>	
<i>rYry</i> $\times$ <i>Ryry</i> -----	1 200	53	38	50	59	0.2
Reciprocal-----	339	65	117	62	95	<.01

<sup>1</sup> Calculated number of plants of each type, 50.

<sup>2</sup> Calculated number of plants of each type, 84.75.

<sup>3</sup> The excess in this class shows distinct evidence of self-fertility.

#### THE FACTORS *R'* FOR RED TOP AND *Y'* FOR ROOT COLOR <sup>13</sup>

##### MATERIAL

The remaining portion of this paper deals with color types obtained from a red beet (origin unknown), the genetic interpretation of which is entirely different from that previously given. The red beet was not observed during its vegetative stage, and consequently its characteristics are known only from the red beet segregates in its progeny. These plants were not so intensely pigmented as the Detroit Dark Red variety, and the petiole pigmentation was confined to a broad stripe which usually did not extend into the midrib or veins of the leaf. These plants were of the type designated as striped red beet (type IV).

The white-root beet originally crossed with the striped red beet was a selection from a Russian sugar beet strain known as F29-01. These parent plants were isolated in 1930 by George Stewart, formerly agronomist at the Utah Agricultural Experiment Station.<sup>14</sup>

<sup>13</sup> Most of the progenies reported in this section were grown by F. V. Owen.

<sup>14</sup> Dr. Stewart left the Utah station in the fall of 1930, turning this material over to the writer, who had been working under his supervision.

The cross of striped red beet  $\times$  red hypocotyl white root yielded only about a dozen seeds, which were all from the striped red beet parent. A few  $F_1$  plants were grown in the greenhouse during the winter of 1930-31, and the seedlings obtained were isolated in one group in the field in 1931. The remaining seeds produced a few  $F_1$  plants which were grown in the field the same year. In 1932, when an  $F_2$  population was grown to maturity, a most interesting segregation occurred. Three types not previously encountered were found, each requiring a modification of the  $RY$  explanation previously employed. These types are (1) red top white root (type VI), assumed to be due to a single factor  $R^t$ ; (2) green top yellow root (type VIII), assumed to be due to a single factor  $Y^r$ ; and (3) green top red root (type V), assumed to be due to a combination of  $R$  (factor for red hypocotyl) plus  $Y^r$  (factor for root color). It is interesting that the striped red beet should have resulted from the combination of  $R^t$  (red top) and  $Y^r$  (root color).

#### $F_1$ BACKCROSSES ( $R^tY^r ry \times r y ry$ )

From the original cross of striped red beet  $\times$  red hypocotyl white root, one  $F_1$  plant of genetic constitution  $R^tY^r ry$  was clonally propagated, two pieces of which matured seeds in backcrosses to beets of the yellow-hypocotyl white-root type ( $ry ry$ ). When the progenies from these crosses were grown, segregates included plants of the green-top yellow-root type ( $rY^r$ ) and the red-top white-root type ( $R^t y$ ). The data from these crosses are presented in table 3. They show a close linkage between  $R^t$  and  $Y^r$ , with approximately 7.5 percent crossing over. The linkage is similar to that between  $Rr$  and  $Yy$ , which has already been discussed.

TABLE 3.—Striped red beet ( $R^tY^r ry$ )  $\times$  the double recessive ( $ry ry$ ), and the reciprocals ( $ry ry \times R^tY^r ry$ )

Cross	Total number of plants	Number of plants of indicated phenotype				Percentage cross-over $\pm$ P. E.
		Striped red beet (type IV)	Red top white root (type VI)	Green top yellow root (type VIII)	Yellow hypocotyl white root (type VII)	
		$R^tY^r$	$R^t y$	$rY^r$ <sup>1</sup>	$ry$	
$ry ry \times R^tY^r ry$ .....	230	97	14	5	114	8.4 $\pm$ 1.24
Reciprocal.....	301	145	7	12	137	6.3 $\pm$ .84
$ry ry \times R^tY^r ry$ .....	268	89	9	8	162	6.3 $\pm$ 1.00
Reciprocal.....	205	133	12	11	109	8.7 $\pm$ 1.17

<sup>1</sup> The factor  $Y^r$  could possibly be synonymous with Kajanus'  $G$ .

<sup>2</sup> The excess number of plants suggests that the female parent was rather self-fertile.

#### CROSSES WITH THE GREEN-TOP YELLOW-ROOT TYPE ( $rY^r ry$ )

The genetic constitution of the green-top yellow-root type ( $rY^r$ ) has been demonstrated in the progeny of crosses between it and four other color types. The data are presented in table 4. The various crosses are as follows:

(1) *Green top yellow root* ( $rY^r ry$ )  $\times$  *yellow hypocotyl white root* ( $ry ry$ ).—The expected ratio in this cross is 1 green top yellow root ( $rY^r$ ) to 1 yellow hypocotyl

white root ( $ry$ ), which is in close agreement with the observed ratio,  $P$  falling between 0.8 and 0.9 (table 4, cross 1).

(2) *Green top yellow root* ( $rYry$ )  $\times$  *red top white root* ( $R'ry$ ).—In this cross the recombination types from the  $F_1$  backcross populations are reunited and the parental types are recovered (table 4, crosses 2 and 3). The first population contains an excess number of plants of the green-top yellow-root type, suggesting some self-fertility in the female parent.

TABLE 4.—Crosses with the green-top yellow-root type ( $rYry$ )

Cross no.	Cross	Total number of plants	Number of plants of indicated phenotype							$P$ from $\chi^2$ test
			Striped red beet (type IV)	Red top white root (type VI)	Red hypocotyl white root (type I)	Green top red root (type V)	Yellow beet (type IX)	Green top yellow root (type VIII)	Yellow hypocotyl white root (type VII)	
			$R'Y'$	$R'y$	$Ry$	$RY'$	$rY$	$rY'$	$ry$	
1	$rYry \times rYry$	192	—	—	—	—	—	47	45	0.80–0.90
2	$rYry \times R'ry$	254	14	7	—	—	—	21	12	.02–.05
3	Reciprocal	384	17	21	—	—	—	24	22	.70–.80
4	$rYry \times rYry$	433	—	—	—	—	15	8	10	.70–.80
5	Reciprocal	576	—	—	—	—	32	24	20	.30–.50
6	$Ryry \times rYry$	154	—	—	38	36	—	45	35	.50–.70

<sup>1</sup> Calculated number of plants in each of the 2 types, 46.

<sup>2</sup> Calculated number of plants in each of the 4 types, 13.5.

<sup>3</sup> Calculated number of plants in each of the 4 types, 21.

<sup>4</sup> Calculated number of plants in the 3 types 16.5, 8.25, and 8.25, respectively.

<sup>5</sup> Calculated number of plants in the 3 types 38, 19, and 19, respectively.

<sup>6</sup> Calculated number of plants in each of the 4 types, 38.50.

(3) *Green top yellow root* ( $rYry$ )  $\times$  *yellow beet* ( $rYr$ ).—The expected ratio in this cross is 2 yellow beet ( $rY$ ) to 1 green top yellow root ( $rY'$ ) to 1 yellow hypocotyl white root ( $ry$ ), and the expected ratios are in good agreement with those observed (table 4, crosses 4 and 5).

(4) *Red hypocotyl white root* ( $Ryry$ )  $\times$  *green top yellow root* ( $rYry$ ).—The progeny from this cross should segregate in the ratio of 1 red hypocotyl white root ( $Ry$ ) to 1 green top yellow root ( $rY'$ ) to 1 green top red root ( $RY'$ ) to 1 yellow hypocotyl white root ( $ry$ ). The actual segregation observed agrees very well with the theoretical ratio,  $P$  being between 0.5 and 0.7 (table 4, cross 6).

The data (table 4) apparently warrant the conclusion that the green-top yellow-root type ( $rY'$ ) is due to a single factor  $Y'$ , which is dominant over the yellow-hypocotyl white-root type ( $ryry$ ).

#### CROSSES WITH THE RED-TOP WHITE-ROOT TYPE ( $R'ry$ )

The red-top character was found to be dependent upon a single factor ( $R'$ ). In backcrosses to the double recessive ( $ryry$ ) a ratio of 1 red top white root ( $R'y$ ) to 1 yellow hypocotyl white root ( $ry$ ) is expected. One progeny gave a ratio of 91 to 86, with  $P$  at 0.7; another gave 24 to 26, with  $P$  between 0.7 and 0.8; and a third gave 59 to 23, but the reciprocal of this cross ( $ryry \times R'ry$ ) gave 15 to 10, with  $P$  at 0.3. The ratio of 59 to 23 was probably due to self-fertility, since the mother plant in the last cross was found to be rather self-fertile; upon selfing, it produced a progeny of 42 individuals in the ratio of 2  $R'y$  to 1  $ry$ . The theoretical ratio for a single factor is 3 to 1, but the total number was small and the agreement proved to be satisfactory,  $P$  falling between 0.2 and 0.3.

Another cross, that of red top white root ( $R'y$ )  $\times$  green top yellow root ( $rY^r$ ), and the reciprocal also support the single factor ( $R^i$ ) concept. This cross has already been discussed and the data presented in table 4, crosses 2 and 3.

CROSSES WITH THE GREEN-TOP RED-ROOT TYPE ( $RyrY^r$ )

Two factors,  $R$  (red hypocotyl) and  $Y^r$  (yellow root) in combination, produce the green-top red-root type ( $RY^r$ ). This was demonstrated in the cross of red hypocotyl white root ( $Ry$ )  $\times$  green top yellow root ( $rY^r$ ) which has already been discussed (table 4, cross 6). Three additional crosses are presented in table 5 in further support of this arrangement. A single plant of the green-top red-root type ( $RY^r$ ) was used as the female parent in all three crosses. This plant was found to be of the genotype  $RyrY^r$ . The linkage between  $Rr$  and  $Y^ry$  appears to be similar to that between  $Rr$  and  $Yy$ , and consequently the calculated populations are based on a gametic ratio of 1 to 12. This linkage relationship also suggests that the factors for red and yellow, respectively, form two multiple allelomorphic series. Crosses with the green-top red-root type were made as follows:

(1) *Green top red root ( $RyrY^r \times$  yellow hypocotyl white root ( $ryry$ ).*—The data from this cross are presented in table 5, cross 1. These data constitute conclusive evidence of close linkage in the repulsion phase. Three other similar crosses were studied, but in the progeny of each some striped red beets appeared. There is good reason to believe that these latter crosses were contaminated, probably because of a mistake in harvesting or in labeling the paper bags.

TABLE 5.—Crosses with the green-top red-root type ( $RyrY^r$ ), with calculated populations based on a gametic ratio of 1 to 12

Cross no.	Cross	Total number of plants	Number of plants in indicated phenotype												Percentage cross-over ± P.E.	P from χ <sup>2</sup> test		
			Red beet (type II)		Striped red beet (type IV)		Red top white root (type VI)		Red hypo-cotyl white root (type I)		Green top red root (type V)		Green top yellow root (type VII)				Yellow hypo-cotyl white root (type VIII)	
			RY		R <sup>i</sup> Y <sup>r</sup>		R <sup>i</sup> y		Ry		RY <sup>r</sup>		rY <sup>r</sup>				ry	
			Observed	Calculated	Observed	Calculated	Observed	Calculated	Observed	Calculated	Observed	Calculated	Observed	Calculated			Observed	Calculated
1	RyrY <sup>r</sup> × rryy	682	-----	-----	-----	-----	-----	-----	-----	23	-----	307	-----	37	-----	8.8 ± 0.73	0.02-0.05 0.05-0.10	
2	RyrY <sup>r</sup> × R <sup>i</sup> Yry	88	-----	-----	16	22	28	22	315	-----	5	1.69	20	20.31	1			1.69
3	RyrY <sup>r</sup> × rYry	97	28	24.25	-----	-----	-----	-----	28	22.38	1	1.87	136	46.63	4			1.87

<sup>1</sup> Plants of the yellow beet type ( $rY$ ) are included because of difficulties in classification, as explained in the text.

<sup>2</sup> May include some of type  $rY^r$ .

(2) *Green top red root ( $RyrY^r \times$  red top white root ( $R^iYry$ ).*—The progeny from this cross segregated into six phenotypes, with  $P$  falling between 0.02 and 0.05, but by assuming a slight amount of self-fertilization a satisfactory fit is obtained (table 5, cross 2).

(3) *Green top red root ( $RyrY^r \times$  yellow beet ( $rYry$ ).*—The data obtained from this cross are presented in table 5, cross 3. The agreement between the observed and expected populations is satisfactory,  $P$  falling between 0.05 and 0.1. This

population was harvested before a means of intensifying the pigment by exposure to sunlight was known; consequently, the two yellow classes are not separated. For the same reason, it is quite possible that some of the plants in the *ry* class should be in the yellow class, which would tend to improve the agreement between the observed and expected populations.

### DISCUSSION

This paper reports a study of the inheritance of the major factors for red and yellow pigment in beets. The nine color types obtained from the progeny of two crosses are shown in table 6.

TABLE 6.—Color types obtained from the progeny of two crosses

Type no.	Type name	Pheno- type	Type no.	Type name	Pheno- type
I.....	Red hypocotyl white root.....	<i>Ry</i>	VI.....	Red top white root.....	<i>R'y</i>
II.....	Red beet.....	<i>RY</i>	VII.....	Yellow hypocotyl white root.....	<i>ry</i>
III.....	Pale red beet.....	<sup>1</sup> <i>RY</i>	VIII.....	Green top yellow root.....	<i>rY'</i>
IV.....	Striped red beet.....	<i>R'Y'</i>	IX.....	Yellow beet.....	<i>rY</i>
V.....	Green top red root.....	<i>RY'</i>			

<sup>1</sup> The writer was unable to determine the nature of the "additional influence" necessary to explain the light color of this type in contrast to the dark-red color of type II.

The investigations reported herein included a study of the progeny of a sugar beet with a red hypocotyl and a white root  $\times$  Detroit Dark Red (an intensely red commercial variety of table beet). In this cross a close linkage with approximately 7.5 percent crossing over is noted between a factor for red hypocotyl (*R*) and a factor for yellow (*Y*). These two factors in combination (*R* plus *Y*) give rise to a red beet of the Detroit Dark Red type, and the absence of both *R* and *Y* gives the double recessive yellow-hypocotyl white-root type (*r* plus *y*).

This paper deals also with the progeny of a striped red beet (origin unknown)  $\times$  a red-hypocotyl sugar beet having a white root. Segregation in the  $F_2$  of this cross gave several plants with red tops and white roots, others with green tops and yellow roots, and one plant with a green top and a red root. Most of the data were satisfactorily explained by assuming the red-top white-root type to be due to a single factor (*R'*), the green-top yellow-root type to be due to a single factor (*Y'*), the striped red beet to be due to a combination of *R'* and *Y'*, and the green-top red-root type to be due to a combination of *R* and *Y'*.

The linkage relation between *R'* and *Y'* is similar to that between *R* and *Y*, and it is thought that the factors for red pigment, *R* and *R'* (plus the recessive *r*), form one allelomorphic series and the factors for yellow pigment, *Y*, *Y'*, and *y* another series. Additional proof for this relationship is desirable, however, since data are not available on the linkage relationship between *R'* and *Y*.

It is of special interest to note that *R* (factor for red hypocotyl), which is necessary for the expression of red color in the Detroit Dark Red variety, is widely distributed in commercial sugar-beet varieties. Therefore, the factor *Y* is the chief consideration in explaining the genetic constitution of the Detroit Dark Red variety, *Y* producing abundant yellow pigment and *R* changing the color to red. In con-



trast to this arrangement, the striped red beet is a combination of a factor ( $Y^r$ ) for pigment in the root plus a factor ( $R'$ ) which produces red in the tops.

The factor  $R'$  is dominant to  $R$  and increases pigment development in the top. Likewise,  $Y$  is considered dominant to  $Y^r$  and increases the abundance of pigment in the petioles and leaves, as well as causing pigment development in the root.

#### SUMMARY

The inheritance of color in nine types of beets obtained from crosses between sugar beets and red beets is reported.

One cross, between a red-hypocotyl white-root sugar beet ( $Ryry$ )  $\times$  Detroit Dark Red table variety ( $RYRY$ ), yielded four types:  $RY$  (red beet);  $Ry$  (red hypocotyl white root);  $rY$  (yellow beet); and  $ry$  (yellow hypocotyl white root). There is conclusive evidence of a close linkage between  $Rr$  and  $Yy$ , with approximately 7.5 percent crossing over. A pale red beet of similar genetic constitution was observed, but its complete genetic make-up for color was not learned.

A second cross between a striped red beet of unknown origin ( $R'Y'R'Y^r$ )  $\times$  a red-hypocotyl white-root sugar beet ( $Ryry$ ) yielded five types:  $R'Y^r$  (striped red beet);  $R'y$  (red top white root);  $rY^r$  (green top yellow root);  $RY^r$  (green top red root); and  $ry$  (yellow hypocotyl white root). The root color was noticeably less intense whenever  $Y^r$  replaced  $Y$  in the various genotypes. There is a close linkage, with approximately 7.5 percent crossing over, between  $R'$  and  $Y^r$  and a similar linkage between  $R$  and  $Y^r$ .

It is believed that the  $R$  factors ( $R$ ,  $R'$ , and  $r$ ) constitute one multiple allelomorph series and the  $Y$  factors ( $Y$ ,  $Y^r$ , and  $y$ ) another series.

# THE RELATION OF PAUSES TO RATE OF EGG PRODUCTION<sup>1</sup>

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## INTRODUCTION

Winter pause is an important character in the limitation of winter egg production during the pullet year. A great deal of the variability in winter production can be accounted for by pauses, differential winter rate, and sexual-maturity factors. The nature of winter pauses has been investigated by Hays (1, 2, 3)<sup>2</sup> and Hays and Sanborn (4, 5, 6), and both hereditary and environmental causes have been recognized (2). The present study was made to determine whether or not pausing bears any relation to the rate of production and to evaluate the accuracy of some methods for its calculation. Rate of production will hereafter be referred to as "rate" for the sake of simplicity.

Hays and Sanborn (5), in presenting different measurements of rate, state that, although the most intimate correlation of winter production with any of the four measures of intensity which they discuss is found to exist with the "net winter rate of laying", "mean size of winter clutch" is the measure to be preferred. In a later paper (6) the same authors claim that the average yearly clutch size is a more accurate criterion of intensity than any short-time measure. "The net winter rate of laying" is defined as the quotient of the total number of eggs from the first pullet egg to March 1 divided by the number of days in the same period less nonproductive periods of 4 or more consecutive days from November 1 to March 1.

Jull (7) proposes a different measure. For evaluation of rate, he uses the number of eggs in the same period studied by Hays and Sanborn expressed as percentage of the total number of days involved. It may be seen that the fundamental difference in the viewpoints of Jull and of Hays and Sanborn lies in the fact that the former evidently ascribes the occurrence of winter pauses to low rate, while the latter consider pause and rate to be separate factors. Knox, Jull, and Quinn (8) also state that the best measure of rate is the production to March 1 as percentage of total number of days involved.

A review of the available literature shows that no evidence as to the nature of a parallel condition of pause during the spring months has been presented. It is probably of some interest to determine whether or not spring pause is related to winter pause, as well as to investigate the relationship of these conditions to rate.

Such a study was undertaken with the foregoing points in view. Statistical constants and correlation of various factors in pausing and nonpausing groups of birds were calculated in the hope of throwing further light on the question.

<sup>1</sup> Received for publication June 13, 1935; issued February, 1936.

<sup>2</sup> Reference is made by number (italic) to Literature Cited, p. 47.

## METHODS

Two periods of production were studied, namely, the winter period, extending from November 1 to February 28, and the spring period, from March 1 to June 28, each exactly 120 days long.

In order to exclude the effect of pausing, broodiness, and late maturity, the rate of production was defined as the number of eggs laid during a period multiplied by 100 and divided by the number of days in the period (120), less the number of days paused and broody. The definition of pause was adopted arbitrarily as 7 or more consecutive nonproductive days. In the case of winter rate, the denominator was further reduced by subtraction of the number of days from November 1 to the date of the first egg, when the latter fell after November 1. This measurement is similar to the one called by Hays and Sanborn (5) net winter rate of laying. It may be seen that the conception of rate introduced here is more closely related to the measure of rate by the mean clutch size, as proposed by Hays (2), than to the method of Jull (7), which does not take nonproductive periods into consideration when calculating rate.

The birds included in this study were selected from the general trap-nested Single Comb White Leghorn flock of the Poultry Division hatched in March and April, on the basis of the following considerations: (1) Only birds which were alive on October 1 of the year following the year of hatch were used; (2) birds which produced less than 30 eggs in either of the two periods studied were eliminated; (3) only birds which commenced laying on or before November 15 of the pullet year were included in the group studied.

Thus the population used was a strictly selected one, and it must be understood that any conclusions arrived at are applicable only to birds conforming to the standards set above. It was felt that a better understanding of the nature of pauses and their relation to the rate of production could be obtained if the study were restricted to birds of the foregoing type. The reasons for this are fairly obvious: Requirement 1 aims to eliminate from consideration the birds which, although apparently healthy, were under the influence of some pathological condition which led to their death before the expiration of the first laying year. Requirement 2 was supplementary to the first one, in the sense that it tended to eliminate birds with abnormal production records. Although in the third year of study birds of that type were killed and examined for evidence of pathological conditions, and were thus eliminated by requirement 1, in the first 2 years no such autopsies were made. The second requirement was therefore introduced to guard against inclusion of diseased birds. Finally, the third requirement served to eliminate part of the possible influence exercised by differential sexual maturity on the factors studied.

## PRESENTATION OF DATA

The total number of birds answering the above requirements in the 3 years of study was 578. These were divided into 4 groups within each year on the basis of the pauses that they exhibited. Thus 12 groups were obtained altogether, 3 groups of each of the following: Birds which paused during the winter period only (WP group), birds which paused only in the spring (SP group), birds which paused both during the winter and spring (WSP group), and birds which did

not pause at all (NP group). The distribution of these 4 groups for each of the 3 years (F, G, and H series, respectively) is shown in table 1. A gradual change in the proportion of pausing and non-pausing birds can be seen from the figures presented.

TABLE 1.—Birds used and percentage distribution on a pause basis

Pause	F series, hatched in 1931		G series, hatched in 1932		H series, hatched in 1933	
	Number	Percent	Number	Percent	Number	Percent
Winter.....	116	58.0	104	46.4	54	35.1
Spring.....	11	5.5	20	8.9	13	8.4
Winter and spring.....	45	22.5	44	19.6	16	10.4
Nonpause.....	28	14.0	56	25.0	71	46.1
Total, 578 birds.....	200	100.0	224	99.9	154	100.0

The comparatively rapid drop in the percentage of winter-pausing birds, as contrasted with the slow decrease in proportion of the spring-pausing group affords the first indication that the two conditions are separate. A further indication of this was obtained by calculating the coefficients of association between winter and spring pause (9, p. 38). These coefficients for the 3 years were found to be  $-0.006$ ,  $+0.085$ , and  $+0.236$ , respectively, indicating that any association present is due to mere fluctuations of sampling.

TABLE 2.—Statistical constants for production and rate of the F, G, and H series of birds shown in table 1

Production or rate	WP group <sup>1</sup>		SP group <sup>1</sup>	
	Mean	Standard deviation	Mean	Standard deviation
Series F:				
Winter production.....	49.930±0.751	11.99±0.53	72.680±1.903	9.36±1.35
Spring production.....	89.330±.645	10.30±.45	69.950±3.285	16.16±2.33
Winter rate.....	62.300±.533	8.52±.37	61.090±.951	4.68±.67
Spring rate.....	74.975±.536	8.56±.38	69.275±1.858	9.14±1.32
Series G:				
Winter production.....	55.260±.982	14.85±.70	76.000±1.671	11.08±1.19
Spring production.....	88.920±.726	10.99±.52	69.000±2.359	15.64±1.67
Winter rate.....	64.755±.583	8.82±.41	64.000±1.315	8.72±.93
Spring rate.....	76.615±.554	8.38±.39	70.250±1.927	12.78±1.37
Series H:				
Winter production.....	60.430±1.339	14.59±.95	85.845±2.760	14.76±1.95
Spring production.....	89.310±1.160	12.73±.83	58.350±3.325	17.78±2.35
Winter rate.....	68.200±.378	4.12±.27	71.130±1.150	6.15±.81
Spring rate.....	76.170±.536	5.84±.38	72.770±1.122	6.00±.79
Production or rate	WSP group <sup>1</sup>		NP group <sup>1</sup>	
	Mean	Standard deviation	Mean	Standard deviation
Series F:				
Winter production.....	49.610±1.414	14.06±1.00	74.500±1.320	10.35±0.93
Spring production.....	69.610±1.400	13.92±.99	90.210±1.047	8.21±.74
Winter rate.....	61.780±.794	7.89±.56	63.070±1.000	7.84±.71
Spring rate.....	70.665±.890	8.85±.63	75.570±.678	5.32±.48
Series G:				
Winter production.....	56.090±1.548	15.22±1.10	78.070±.711	7.89±.50
Spring production.....	67.230±1.752	17.23±1.24	88.960±.992	11.01±.70
Winter rate.....	63.705±.890	8.46±.61	66.955±.567	6.29±.40
Spring rate.....	70.865±1.211	11.91±.86	74.770±.798	8.86±.57
Series H:				
Winter production.....	59.500±1.868	11.07±1.32	82.390±.722	9.02±.51
Spring production.....	72.000±2.632	15.61±1.86	91.240±1.316	16.45±.94
Winter rate.....	66.060±1.163	6.90±.82	69.745±.506	6.33±.36
Spring rate.....	72.000±1.635	9.70±1.15	75.240±.594	7.42±.42

<sup>1</sup> WP=winter pause; SP=spring pause; WSP=both winter and spring pauses, NP=no pause

Table 2 presents the statistical constants for the 12 groups of birds for winter production, spring production, winter rate, and spring rate. This table gives the basic data from which differences between groups were calculated. Since there is a possible effect of environment and managemental practices, differences between means were determined only within each series; table 3 presents these. In all of the tables probable and not standard errors are used.

TABLE 3.—*Differences between means of winter and spring production and rates for birds as calculated from data in table 2*

WINTER PRODUCTION			
[Significant differences are indicated in boldface and borderline differences in italic>			
Groups <sup>1</sup>	F series	G series	H series
NP to SP.....	1.820±2.316	2.070±1.816	-3.455±2.853
NP to WP.....	24.570±1.519	22.810±1.212	21.960±1.521
NP to WSP.....	24.890±1.934	21.980±1.703	22.890±2.001
SP to WP.....	22.750±2.046	20.740±1.938	25.415±3.088
SP to WSP.....	23.070±2.371	19.910±2.278	26.345±3.332
WP to WSP.....	.320±1.601	-.830±1.833	.930±2.297
SPRING PRODUCTION			
NP to WP.....	0.880±1.230	0.040±1.229	0.930±1.760
NP to SP.....	20.260±3.448	19.960±2.559	31.880±3.578
NP to WSP.....	20.600±1.748	21.730±2.013	18.240±2.943
WP to SP.....	19.350±3.349	18.920±2.468	30.960±3.525
WP to WSP.....	19.720±1.541	21.690±1.897	17.310±2.880
SP to WSP.....	.340±3.571	1.770±2.939	-15.650±4.241
WINTER RATE			
NP to SP.....	1.980±1.380	2.955±1.432	-1.385±1.257
NP to WP.....	.770±1.133	2.200±.813	1.545±.632
NP to WSP.....	1.290±1.277	<i>5.250±1.030</i>	3.685±1.268
SP to WP.....	-1.210±1.090	-1.755±1.438	2.930±1.211
SP to WSP.....	-.690±1.239	.295±1.571	<i>5.070±1.656</i>
WP to WSP.....	.520±.956	1.050±1.039	2.140±1.223
SPRING RATE			
NP to WP.....	0.595±.864	-1.845±.969	-0.930±.800
NP to SP.....	<i>6.295±1.973</i>	4.520±2.086	2.470±1.270
NP to WSP.....	4.905±1.119	3.905±1.450	3.240±1.740
WP to SP.....	5.700±1.934	<i>6.365±2.005</i>	3.400±1.243
WP to WSP.....	4.310±1.039	5.750±1.332	4.170±1.720
SP to WSP.....	-1.390±2.060	-1.615±2.276	.770±1.983

<sup>1</sup> For explanation of symbols see footnote to table 2.

Table 3 shows that, as would be expected, occurrence of a pause or pauses in the winter period reduced the winter egg production by 19.9 to 26.3 eggs in the various groups. The groups which showed spring pause did not differ significantly in winter production from the non-spring-pause groups. Likewise there was no significant difference in winter production between the winter-pause birds and those birds which besides the winter pause also exhibited a spring pause.

Similarly, table 3 shows that spring production is reduced by the occurrence of spring pause by 17.3 to 31.9 eggs, while winter pause does not seem to have any effect on the spring production. The only exception to this is the case of the winter- and spring-pause group of

the H series. While still significantly below the nonpause and winter-pause groups in spring production, this group shows a mean spring production higher by 13.65 eggs than the group which paused during the spring only. This figure is slightly more than three times its probable error and as such approaches the borderline limit of statistical significance.

Table 3, which also shows differences in winter rate between the various groups, is of interest since it discloses that winter rate as here calculated is not affected by the occurrence of winter pause. Only two of the differences at all approach statistical significance and even these are less than 3.5 times the probable error. In 3 of the 12 cases, the group showing winter pause has a higher winter rate than the corresponding nonpause groups. This seems to be an indication that winter pause should not be considered merely as a manifestation of low rate.

So far as spring pause is concerned, it may be seen from table 3 that in some cases this condition appears to affect the rate of spring production. Of the 12 differences in spring rate between the groups not pausing in the spring and the groups exhibiting a spring pause, 3 are statistically significant while 2 more are below 3.5 but above 3.0 times their respective probable errors. There is, therefore, a possibility of an association between spring rate and spring pause. The coefficients of association between low spring rate and spring pause were found to be  $+0.492$ ,  $+0.344$ , and  $+0.286$  for the F, G, and H series, respectively. Thus it seems that there is a difference in the nature of the winter and spring pauses. Should any genetic interpretation of winter pause, such as that of Hays (1), be accepted, one must perforce conclude that the spring-pause condition, if genetic in nature, is governed by factors other than those controlling winter pause.

In order to determine whether or not pauses have an effect on the production in periods immediately preceding and immediately following them, the rates of production for the 15 days before and for the 15 days after a pause were calculated. These values are presented in table 4 together with the rate for the total winter or spring

TABLE 4.—Rate of production for 15 days preceding and for 15 days following a pause

Group <sup>1</sup>	Winter rate			Spring rate		
	Pre-pause	Total period	Post-pause	Pre-pause	Total period	Post-pause
Series F:						
WP	58.96	62.30	58.83			
SP				61.23	69.28	77.81
WSP	57.23	61.78	58.96	68.47	70.67	67.40
Series G:						
WP	60.70	64.76	54.83			
SP				69.33	70.25	66.67
WSP	58.81	63.71	50.86	68.23	70.87	65.17
Series H:						
WP	64.70	68.20	67.27			
SP				73.34	72.27	62.23
WSP	62.65	66.06	66.23	69.60	72.00	67.54

<sup>1</sup>For explanation of symbols see footnote to table 2.

period, as the case may be. The rate both before and after a pause seems to be somewhat depressed except in a few cases. This reduction in rate preceding and following the pause may be an expression of the factors producing the pause and not a true manifestation of factors for rate, at least in the case of winter rate, since, as shown in table 3, the winter rate of pausers for the whole period does not differ significantly from the winter rate of nonpausers.

### COEFFICIENTS OF CORRELATION

Coefficients of correlation between the four factors studied were computed and are presented in table 5. The most significant points to be noted from these correlations are:

(1) The coefficients of correlation between winter rate and winter production are, as expected, somewhat higher in groups which did not pause in the winter than in groups which did.

TABLE 5.—Coefficients of correlation in the different series as calculated from the data in table 3

Factors correlated	F series	G series	H series
Group WP: <sup>1</sup>			
Winter production and winter rate.....	0.288±0.058	0.460±0.052	0.634±0.055
Winter production and spring production.....	.101±.063	.227±.063	.445±.074
Winter production and spring rate.....	.179±.061	.327±.059	.388±.078
Winter rate and spring production.....	.327±.056	.478±.051	.481±.075
Winter rate and spring rate.....	.422±.052	.572±.044	.592±.060
Spring production and spring rate.....	.839±.019	.812±.021	.818±.030
Group SP: <sup>1</sup>			
Winter production and winter rate.....	.897±.040	.927±.021	.955±.016
Winter production and spring production.....	.726±.096	.375±.130	.122±.184
Winter production and spring rate.....	.367±.176	.548±.106	.411±.155
Winter rate and spring production.....	.660±.114	.411±.126	.149±.183
Winter rate and spring rate.....	.261±.189	.626±.086	.381±.160
Spring production and spring rate.....	.624±.124	.828±.047	.477±.144
Group WSP: <sup>1</sup>			
Winter production and winter rate.....	.411±.084	.614±.064	.758±.063
Winter production and spring production.....	.241±.095	.112±.101	.253±.139
Winter production and spring rate.....	.026±.101	.392±.086	.058±.149
Winter rate and spring production.....	.354±.088	.059±.102	.225±.141
Winter rate and spring rate.....	.354±.088	.363±.080	.211±.142
Spring production and spring rate.....	.604±.064	.746±.045	.888±.031
Group NP: <sup>1</sup>			
Winter production and winter rate.....	.881±.028	.896±.018	.720±.039
Winter production and spring production.....	.294±.110	.434±.073	.367±.069
Winter production and spring rate.....	.389±.107	.472±.070	.376±.069
Winter rate and spring production.....	.322±.114	.443±.072	.573±.054
Winter rate and spring rate.....	.401±.107	.455±.071	.531±.057
Spring production and spring rate.....	.882±.028	.927±.013	.965±.006

<sup>1</sup> For explanation of symbols see footnote to table 2.

(2) The correlation coefficients between spring rate and spring production are higher in the nonspring-pause groups than in the spring-pause groups, although the differences here are not so marked as in the case of the winter period. The occurrence of broody periods in the summer months in groups which did not show any pause is probably responsible for this.

(3) The magnitude of the correlation coefficients between winter and spring production varies from  $+0.101 \pm 0.063$  for the WP group of the F series to  $+0.726 \pm 0.096$  for the SP group of the same series. No particular regularity can be observed in the variation of this coefficient.

(4) The correlation between winter and spring rate also shows some variation, the lowest coefficient being  $+0.211 \pm 0.142$  for the WSP

group of the H series, and the highest  $+0.626 \pm 0.086$  for the SP group of the G series. It is a fact of great importance that, in all cases except two, winter rate is more closely correlated with spring rate than is winter production with spring production. The two exceptions represent populations of 11 (SP group, F series) and of 16 (WSP group, H series) respectively. It should also be noted that the correlation between spring rate and winter rate may be somewhat reduced in the total population as a result of spring pause.

Figure 1, based on the total population of 578 birds, illustrates the regression of spring rate on winter rate. It is definitely linear, the difference between the correlation ratio and correlation coefficient being, according to Blakeman's test,  $0.017 \pm 0.007$ . Hays and Sanborn (6), however, found that the correlation between winter- and spring-clutch size was curvilinear to a small degree.

It may be seen from the slope of the line in figure 1 that the percentage of improvement in spring rate over winter rate is greater in the birds with the lower winter rate. Birds exhibiting a winter rate of about 80 percent and over do not show any increase of rate in the spring, and may even exhibit a decline. On the whole, it may

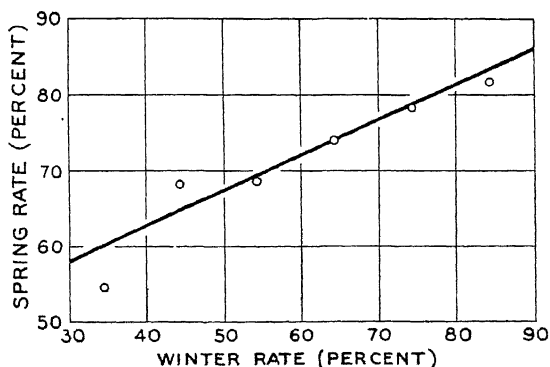


FIGURE 1.—Regression of spring rate on winter rate;  $SR = 44.256 + 0.463 WR$ .

be seen that there is a possibility that the winter and the spring rates are governed by the same genetic factors, their expression depending on environmental conditions.

It may be reiterated here that two views on the matter are possible. One view, that of the Beltsville workers (7, 8), is that pauses are a reflection of a low rate, the other, held by Hays and Sanborn (5, 6), is that winter pause is a separate entity. The evidence presented here may be interpreted to mean that pauses, whether genetically or environmentally determined, should be excluded in any method of measuring rate.

The effect of inclusion of pause periods in the calculation of rate may be observed in table 6. Series G is used as the one with the largest number of individuals. The winter and spring rates are shown for the various groups as calculated by two methods. The Beltsville method consists in dividing the number of eggs in a period  $\times 100$  by the total number of days, while in the California modification of the Massachusetts method the days of pause and of broodiness are first subtracted from the denominator.



TABLE 6.—Comparison of methods of measuring rate of production in various groups<sup>1</sup> of the G series by Beltsville and California methods

Rate and method	WP group	SP group	WSP group	NP group
Winter:				
Beltsville.....	46.05	63.33	46.71	65.06
California.....	64.76	64.00	63.71	66.96
Spring:				
Beltsville.....	74.10	57.50	56.03	74.13
California.....	76.62	70.25	70.87	74.77

<sup>1</sup> For explanation of symbols see footnote to table 2.

According to the Beltsville method, the birds in the WSP and the NP groups are, respectively, low and high for both periods. In the WP group the birds are low for winter rate but high for spring rate, while in the SP group the reverse is true. On the other hand, according to the California method, the birds in each of the groups show the same type of rate irrespective of the season for which rate is calculated. By using the regression line in figure 1 it is then possible to approximate the rate of one period from information on the rate of the other. If rate is a character controlled by the same factors throughout the year, this evidence indicates that there is no justification for any method of measuring rate which does not exclude the pausing periods from consideration.

The absence of any relationship between winter rate as here calculated and some of the other factors which may affect production is shown by the following coefficients of correlation (WP group, G series) between—

Winter rate and—

Date of hatch.....	-0.011 ± 0.066
Date of first egg.....	-.005 ± .066
Age at first egg.....	-.046 ± .066
Winter pause length.....	-.078 ± .066

While Hays (3) has found that his nonbroody line of Rhode Island Reds had a mean winter production higher by about 12 eggs than his broody line, in the case of the Single Comb White Leghorns here studied, there was no difference between broody and nonbroody birds in rate of winter production, but there was some difference in spring rate. Table 7, illustrating this point, shows that the broody birds had a higher rate of spring production than the nonbroody birds. Because of the small number of broody birds, it is not possible to check the significance of this observation by the usual methods.

TABLE 7.—Rate of production of broody and of nonbroody birds

Series or group <sup>1</sup>	Total population	Broody	Winter rate		Spring rate	
			All birds	Broody birds	All birds	Broody birds
		<i>Number</i>				
F.....	200	11	62.225	62.400	73.775	75.091
WP in G.....	104	10	64.755	64.400	76.615	80.500
G.....	224	25	64.745	66.640	74.480	80.320
H.....	154	8	68.980	69.500	76.280	80.500

<sup>1</sup> For explanation of symbols see footnote table 2.

## CONCLUSIONS AND SUMMARY

A study of the relation of pauses to the rate of egg production was made on a selected population of 578 Single Comb White Leghorns in their pullet year. The birds were selected for absence of pathological conditions and for fairly uniform early sexual maturity. Three series of birds, hatched in 3 consecutive years, were represented in the total population and each series was treated separately. Analyses of differences in the rate of production between pausing and nonpausing birds, as well as a study of correlations between rate and number of eggs laid during different periods led to the following major conclusions:

(1) The condition of winter pause is causally distinct from that of spring pause and is entirely separate from it.

(2) The view that winter pause is merely a manifestation of low winter rate is untenable.

(3) Spring pause may be associated with low spring rate, but no conclusive evidence on this point is as yet available.

(4) Spring rate shows a linear regression on winter rate and an increase over it when the latter is below 80 percent. Evidence on hand points to the possibility that the same genetic factors control both winter and spring rate of production.

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# THE EFFECT OF HOMOGENIZATION ON SOME OF THE CHARACTERISTICS OF MILK FAT<sup>1</sup>

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## INTRODUCTION AND REVIEW OF LITERATURE

The adaptation of the homogenization process to the market-milk industry has been confined largely to pasteurized milk, since raw milk was found to develop a rancid flavor within a few hours after being homogenized. The rapid development of rancidity in homogenized raw milk and the absence of a similar development in milk pasteurized prior to homogenization has been noted by several investigators, including Dorner and Widmer (5),<sup>3</sup> Halloran and Trout (6), Doan and Minster (4), and Babcock (2). Dorner and Widmer (5) attributed the development of the rancid flavor to lipase action resulting in the splitting of the fat to give free fatty acids. This view has been generally accepted by other investigators. The associates of Rogers (8) had earlier pointed out that the production of free fatty acids in some dairy products is due primarily to hydrolytic action of enzymes or the action of free acids in the product in the presence of water. These authors state further that the production by hydrolysis of some of the lower fatty acids, such as butyric, caproic, and others, are responsible for the rancid flavor and odors.

Dorner and Widmer (5), and Halloran and Trout (6), working independently, observed an increase in titratable acidity of homogenized raw milk, the increase appearing immediately after homogenization and varying directly with the pressure used. Pasteurization before homogenization prevented the occurrence of any increase in acidity. Doan and Minster (4) secured similar results by pH measurements.

The decrease in the surface tension of homogenized raw milk, as noted by Halloran and Trout (6), was ascribed by Doan (3) to be the result of the liberation of some of the lower fatty acids from the glyceride, and an increase in their concentration at the surface of the milk.

The associates of Rogers (8), Lewkowitsch (7), and others give the normal ranges of characteristic milk-fat constants, such as refractive index, Reichert-Meissl number, Polenske number, and acid value. However, no information is available regarding the effect of homogenizing either raw or pasteurized milk on these values. According to the former authors, the normal range of the refractive index of milk fat at 40° C. is from 1.4527 to 1.4566. Lewkowitsch (7, *v. 1, p. 124*) studied pure fatty acids and found the indexes of butyric and caproic acids at 20° C. to be 1.39906 and 1.41635, respectively.

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<sup>2</sup> The authors wish to express their appreciation to Prof. C. D. Ball, Chemistry Department, Michigan State College, for valuable suggestions and criticisms relative to this study.

<sup>3</sup> Reference is made by number (italic) to Literature Cited, p. 56.

## SCOPE OF INVESTIGATION

Although suggestions have been made that increases in titratable acidity, decreases in the hydrogen-ion concentration, and development of rancidity in homogenized raw milk were due to changes in the fat, all evidence for these assumptions has been more or less indirect in that the measurements have been made upon the milk rather than upon the fat itself. In this connection it appeared of interest to determine the actual chemical changes in the fat brought about by homogenization both of raw and of pasteurized milk, and to determine the extent to which the fat is changed in the development of rancidity. To secure such information, a study of changes in some of the fat constants appeared to offer the best possibilities. The fat constants chosen for examination were as follows: The Reichert-Meissl number, the Polenske number, the acid degree, and the refractive index.

## PROCEDURE

Raw milk having a fat test of approximately 3.5 percent was used for the experiments. During each of the five trials run, the milk was divided into four lots and processed with commercial equipment, as follows:

Lot 1. Pasteurized at 145° F. for 30 minutes.

Lot 2. Pasteurized at 145° F. for 30 minutes, and then homogenized at 1,500 pounds pressure per square inch.

Lot 3. Warmed to 100° F., homogenized at 1,500 pounds pressure, and immediately thereafter pasteurized at 145° for 30 minutes. About 10 to 15 minutes was usually required to reach the pasteurizing temperature after homogenization.

Lot 4. Homogenized at 100° F., cooled to about 55° over a surface cooler, and then stored at 35° to 40° for 24 hours. After storage, the milk was pasteurized, as in the previous lots.

Following the processing of the lots, samples were taken, cooled, and the pH value and titratable acidity of the milk determined. The pH value was obtained electrometrically with a Leeds-Northrup portable acidity meter using a bright platinum foil electrode and quinhydrone for the determinations. The milk was then separated, and the cream churned. The fat obtained was washed several times with water and melted in a hot-water bath. It was further purified by filtering through a paper filter to eliminate curd and water. The purified fat obtained was used for the determination of the Reichert-Meissl number, the Polenske number, the acid degree, and the refractive index. An Abbé refractometer was used, according to the directions of the Association of Official Agricultural Chemists (1).

## RESULTS

## THE EFFECT OF HOMOGENIZATION ON THE TITRATABLE ACIDITY AND HYDROGEN-ION CONCENTRATION OF THE MILK

As mentioned previously, several investigators observed the effect on the titratable acidity and the hydrogen-ion concentration when raw and pasteurized milk were homogenized. These published results showed that the homogenization of pasteurized milk had no effect on the hydrogen-ion concentration or acidity, whereas the homogenization of raw milk increased the acidity and decreased the hydrogen-ion concentration. A verification of these results was

secured in this study, but the data are not included inasmuch as they are a repetition of what has formerly been thoroughly studied. It suffices to say that in the five trials made, no appreciable differences occurred either in the titratable acidity or in the hydrogen-ion concentration when the milk was homogenized following pasteurization, but a noticeable change in both of these values was observed when the milk was homogenized raw and then pasteurized. These changes were greater when 24 hours had elapsed between homogenization and pasteurization. During this period, the titratable acidity increased from an average of 0.17 to an average of 0.20 percent, calculated as lactic acid, while the hydrogen-ion concentration value decreased from 6.58 to 6.44. The determination of titratable acidity, calculated as lactic acid, appeared to be as accurate as hydrogen-ion concentration measurements in detecting acidity changes in the milk due to homogenization.

THE EFFECT OF HOMOGENIZATION OF MILK ON THE REICHERT-MEISSL AND POLENSKE NUMBERS OF THE FAT

The data secured in the study of the influence of homogenization of raw and pasteurized milk on the Reichert-Meissl and Polenske values of the butterfat are presented in table 1.

TABLE 1.—*The effect of homogenizing raw and pasteurized milk upon the Reichert-Meissl and Polenske numbers, the refractive index, and the acid degree of the butterfat*

REICHERT-MEISSL NUMBER				
Trial no	Pasteurized, unhomogenized	Pasteurized, homogenized	Raw, homogenized	Raw, homogenized, stored 24 hours
1.....	29.68	28.90	30.21	29.12
2.....	31.25	30.94	31.44	31.00
3.....	30.14	30.00	29.81	29.24
4.....	30.97	30.66	30.80	30.65
5.....	28.18	28.11	27.93	27.79
Average.....	30.04	29.72	30.04	29.56
POLENSKE NUMBER				
1.....	3.27	3.27	3.25	3.18
2.....	2.84	2.83	2.93	2.85
3.....	2.07	2.07	1.89	1.82
4.....	2.83	2.99	2.80	2.76
5.....	1.89	2.00	1.92	1.93
Average.....	2.59	2.63	2.56	2.51
REFRACTIVE INDEX				
1.....	1.4542	1.4541	1.4541	1.4540
2.....	1.4552	1.4552	1.4551	1.4551
3.....	1.4552	1.4551	1.4550	1.4551
4.....	1.4552	1.4552	1.4551	1.4548
5.....	1.4551	1.4550	1.4550	1.4552
Average.....	1.4550	1.4549	1.4545	1.4548

TABLE 1.—*The effect of homogenizing raw and pasteurized milk upon the Reichert-Meissl and Polenske numbers, the refractive index, and the acid degree of the butterfat—Continued*ACID DEGREE<sup>1</sup>

Trial no.	Pasteurized, unhomogenized	Pasteurized, homogenized	Raw, homogenized	Raw, homogenized, stored 24 hours
1.....	0.72	0.74	2.65	9.86
2.....	.32	.33	2.55	-----
3.....	.67	.60	2.73	11.20
4.....	.50	.50	2.53	14.40
5.....	.65	.66	2.70	6.12
Average.....	.572	.566	2.608	10.395

<sup>1</sup> Acid degree is expressed as the cubic centimeters of N/1 NaOH required to titrate the free fatty acids in 100 g of fat.

<sup>2</sup> Not included in averages.

The averages of these trials vary but do not show any general trend in changes of these constants. The data show no great differences between the fat from the homogenized raw samples and that from the original unhomogenized pasteurized milk. The Reichert-Meissl and Polenske titrations decreased only 0.48 and 0.08 cc, respectively, in the 24-hour raw sample. Such changes appear negligible.

## THE EFFECT OF HOMOGENIZATION OF MILK ON THE REFRACTIVE INDEX OF THE FAT

The results of the study of homogenization as it affects the refractive index of the milk fat are also presented in table 1. As shown in this table, homogenization of the raw or of the pasteurized milk had no marked influence on the refractive index. A slight decrease in the indexes of the fat from the raw samples is shown by the average of the trials, but the change is so slight as to appear of no value.

## THE EFFECT OF HOMOGENIZATION OF MILK ON THE ACID DEGREE OF THE FAT

Although none of the fat constants reported in table 1 appeared to be affected by homogenization, the acid degrees of the fat were materially changed. Table 1 gives the values secured. The data show that the average acid degree of the fat of the raw homogenized milk immediately after processing was approximately 4 times that of the pasteurized milk, whereas after the raw homogenized milk had been stored 24 hours the average acid degree of the fat was 18 times that of the pasteurized milk.

The acid values secured were practically constant between the two pasteurized samples, indicating that there was no liberation of fatty acids when the milk was pasteurized prior to homogenization.

The acid degree of the fat in the raw homogenized sample in trial no. 3 showed scarcely any increase as it did in the other four trials, and, because of this deviation, the value was not included in the averages.

The values secured for the raw homogenized samples stored 24 hours varied greatly. The exact cause for these variations is not known.

THE EFFECT OF LONG STORAGE PERIODS ON THE TITRATABLE ACIDITY AND HYDROGEN-ION CONCENTRATION VALUES OF THE RAW HOMOGENIZED MILK AND ON THE ACID DEGREE OF THE FAT

Since the acid value was found to be so greatly affected when raw milk was homogenized, it was thought desirable to make a study of the changes in the free acids of the fat when the milk was stored for several days following homogenization. The raw milk was homogenized at 1,500 pounds pressure, cooled immediately over a surface cooler to 55° F., and stored at 35° to 40°. Portions of this milk were taken at daily intervals, pasteurized, and the fat studied. The control sample was pasteurized but not homogenized.

Titratable acidities and hydrogen-ion concentration measurements were also made on the milk after varying periods of storage. The values of these latter determinations are given in table 2 and show a marked increase in titratable acidity and increase in hydrogen-ion concentration in the milk during the 5-day storage period.

TABLE 2.—*The effect of storing for varying periods of time, the titratable acidity, and hydrogen-ion concentration of raw homogenized milk*

Period after homogenization (hours)	Trial no. 1		Trial no. 2		Trial no. 3		Average	
	Acidity	pH	Acidity	pH	Acidity	pH	Acidity	pH
	<i>Percent</i>		<i>Percent</i>		<i>Percent</i>		<i>Percent</i>	
Control.....	0.182	6.42	0.155	6.50	0.145	6.38	0.161	6.43
14-1/2.....	.190	6.38	.170	6.48	.155	6.35	.172	6.40
24.....	.240	6.24	.200	6.28	.185	6.32	.208	6.28
48.....	.245	6.25	.240	6.28	.185	6.32	.223	6.28
72.....	.255	6.19	.235	6.28	.190	6.32	.227	6.26
96.....	.255	6.14	.238	6.20	.195	6.30	.229	6.21
120.....	.265	6.17	.245	6.15	.200	6.26	.237	6.19

The data showing the effect of the varying periods of storage on the acid degree of the fat are presented in table 3. These figures show the tremendous increase of free acids in the homogenized raw milk during each of the storage periods. Even at the end of the fifth day the liberation of the acids continued rapidly.

TABLE 3.—*The effect of storing for varying periods of time on the acid degree of the fat of raw homogenized milk*

Period after homogenization (hours)	Acid degree of the fat				Average increase
	Trial no. 1	Trial no. 2	Trial no. 3	Average	
					<i>Percent</i>
Control.....	0.595	0.645	0.460	0.566	0
14-1/2.....	4.390	3.850	2.515	3.585	533
24.....	11.200	9.960	8.600	9.920	1,652
48.....	14.690	13.370	8.560	12.207	2,056
72.....	16.000	14.980	10.300	13.760	2,331
96.....	18.500	15.200	10.870	14.856	2,524
120.....	18.400	18.010	12.640	16.350	2,788

The greatest daily change in the fatty-acid values occurred during the first 24 hours of storage, the increase amounting to an average of 1,652 percent during this time. Furthermore, the acid value of the



fat increased on an average of 533 percent within a few minutes after homogenization.

The increase in the degree of free acids in the fat, and in titratable acidity, and the decrease of the pH value are shown in figure 1. In this figure the pH values were plotted as hydrogen-ion concentration  $[H^+]$  so as to form a curve which may be compared as to slope with the curve of the acid value.

Figure 1 shows the rapid increase in the free fatty acids during the first 24 hours, or even during the first few minutes, after homogenization. The speed of the splitting of the fatty acids from the glycerol

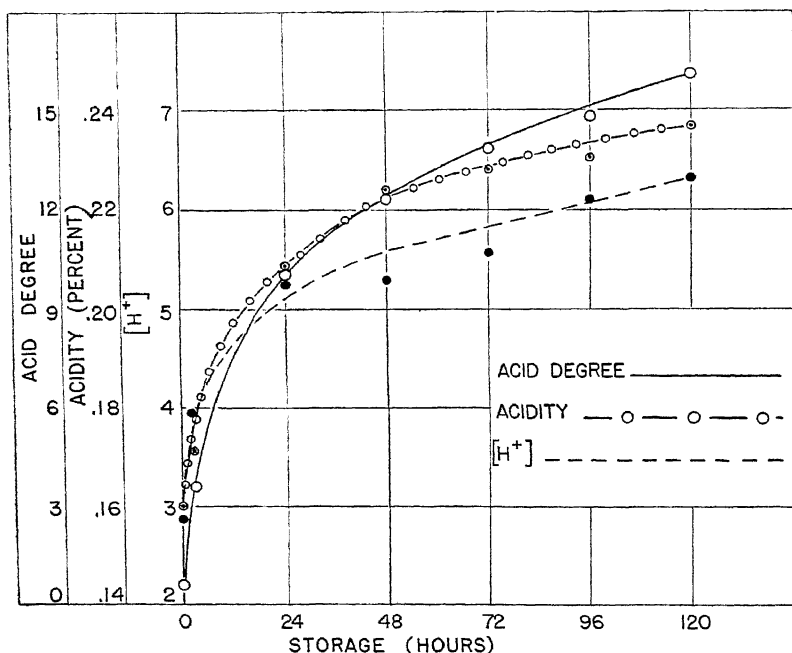


FIGURE 1—Changes in the acid degree of the fat, the titratable acidity, and the hydrogen-ion concentration of raw homogenized milk. The units indicating  $[H^+]$  should be multiplied by  $10^{-7}$  to express accurate concentration. The acidity is expressed as the percentage of lactic acid, and the acid degree as the number of cubic centimeters of N/1 NaOH required to titrate the free fatty acids in 100 g of fat.

by enzymic action apparently proceeds at a high velocity when the fat globules are first broken by the homogenizing process into smaller globules, thus exposing more surface to the splitting action.

Figure 1 also shows that the titratable-acidity measurements of the milk followed increases in free acids of the fat somewhat more closely than the pH values. Since only three trials supplied data for this graph it may be presumptuous to assume that this difference will always occur. It appears safe to say, however, that determinations of rancidity development in milk may be made as satisfactorily with titration means as with potentiometric methods.

## DISCUSSION

Of the four fat constants investigated in the present study only the acid value showed distinct changes as a result of homogenization of raw milk. Homogenization of pasteurized milk did not cause any appreciable changes in the Reichert-Meissl number, the Polenske number, the refractive index, or the acid degree.

If only the liberation of the lower fatty acids occur in the fat of homogenized raw milk, no changes would be expected in the Reichert-Meissl or Polenske numbers since the amount of soluble volatile and insoluble volatile acids would naturally remain constant regardless of whether the acid was in the free or combined state. It is likewise obvious that during the determination of these two constants, the lower fatty acids must necessarily be freed.

A decrease in the refractive index of milk fat might be expected to occur with high concentrations of free acids, when consideration is given to the refractive indices of pure fatty acids. The average refractive index of the fat in this study was 1.4548 at 40° C., whereas Lewkowitsch (7, *v. 1*, *p. 124*) gave a value of 1.39906 at 20° for pure butyric acid. Correcting the milk-fat index of 1.4549 at 40° to 20° by using the temperature correction formula  $R = R' + 0.00038 (T' - T)$  as given by the Association of Official Agricultural Chemists (1), the index of refraction of the fat is increased to 1.4625. This correction makes even larger the difference between the indexes of the butyric acid and the fat, indicating that the presence of any appreciable amount of butyric acid would have a marked tendency to lower the index of refraction of milk fat. In the trials herein reported, however, no great differences were detected even though the acid degree increased on an average from 0.572 to 10.40 during a 24-hour holding period. The averages of the five trials show a slight decrease in the refractive index of the fat in the raw samples, but the decrease is so small as to appear negligible. A greater decrease than this would be expected with an acid degree of 10.37, or the presence of 0.912 percent n-butyric acid.

In this connection, however, the refractive index of the glycerol must not be overlooked. Lewkowitsch (7, *v. 3*, *p. 402*) gives values for pure glycerol secured both by Strohmer and Lenz which are higher than the index values of butterfat. The refractive index of glycerin at 20° C. is approximately 1.4727. Therefore, when butterfat is hydrolyzed, the slightly higher index of refraction of glycerin might tend to balance the lower values of the free fatty acids.

The interesting point in regard to the acid degrees of the fat taken from raw homogenized milk after varying periods of storage is the large increase in this value. The increase on an average of 2,788 percent over the control pasteurized sample after 5 days' storage illustrates the rate at which fat-splitting action proceeds even at low-storage temperatures. Calculations show that the 16.35 cc of N/1 NaOH required to titrate the free acids in 100 g of fat are equivalent to 1.4388 g of butyric acid. Assuming the butyric acid content of butterfat to be 3 percent, these values indicate that about 48 percent of the butyric acid is liberated at the end of the 5-day storage period. This value may be somewhat low if consideration is given to the possibility that some of the glycerol and butyric acid are lost

during the washing of the fat. Likewise, it is likely that other lower fatty acids in addition to butyric are affected by the fat-splitting process.

It is known that the homogenization process increases the surface of the fat globules about 4 to 6 times. The data obtained in this study show that the acid degree of the fat in the raw homogenized samples increased fourfold to sixfold within a few minutes after homogenization of raw milk. Perhaps the entire immediate increase in the acid degree following homogenization may be explained by the increase in the surface of the fat, thus permitting greater fat-splitting activity by the causative agent. Further increases may be due not only to enzymic action but to the presence of the free acids which hasten further hydrolysis of the fat.

From time to time rather distinct differences were observed between the speed with which the acid value increased in samples of milk with the same fat content. These differences may have been partly due to changes in storage temperatures but more likely were principally caused by variations inherent in the milk itself.

#### SUMMARY AND CONCLUSIONS

No appreciable differences in the Reichert-Meissl number, the Polenske number, the refractive index, or in the acid degree occurred in the fat when pasteurized milk was homogenized at 1,500 pounds pressure per square inch.

When raw milk was homogenized, the acid degree of the fat increased fourfold to sixfold within a few minutes.

The normal average acid degree of the fat (expressed in cubic centimeter N/1 NaOH per 100 g of fat) was 0.572. This value increased in raw milk to 2.608 immediately after homogenizing.

The greatest daily change in the acid degree of the fat of stored homogenized raw milk occurred during the first 24 hours when the titration of free acids with N/1 alkali increased on an average from 0.566 to 9.92 cc, a 1,652-percent increase.

Homogenized raw milk, stored for 5 days, required an average of 16.35 cc N/1 NaOH to titrate the free acids in 100 g of fat, or an equivalent of 1.4388 g of butyric acid.

The measurement of free fatty acids by titration of the fat appears to be a more accurate and more sensitive means of determining the rate of fat-splitting action than those determinations which may be made upon the milk.

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# PERIDERM AND CORTEX COLOR INHERITANCE IN THE POTATO<sup>1</sup>

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## INTRODUCTION

Red and blue tuber color in varieties of white-and yellow-fleshed potatoes (*Solanum tuberosum* L.) is due to pigment located in the periderm, in the peripheral cortex, or in both periderm and cortex. Krantz (5)<sup>2</sup> found the location of pigment to be specific for certain progenies. Location of pigment is not always indicated, and in general receives little attention in the contributions of Salaman (10, 11), Asseyeva (1), Sirks (12), Lunden (?), Black (?), and Rathlef (9). McIntosh (8) and Klapp (3) found, in systematic studies of potato varieties, that color of flowers was associated with color of periderm, but not with color of cortex. This paper presents data on the inheritance of periderm color and cortex color and also considers interaction between periderm and cortex color factors.

## INHERITANCE OF RED COLOR IN THE PERIDERM

Color in the periderm (the cork or skin) may usually be recognized in portions of the periderm separated by light scraping from the underlying tissue of immature tubers.

Previous observations (4, 5, 6) have aided the formulation of a working hypothesis which assumes that red periderm color is due to the complementary action of three factors *E*, *S*, and *F*.

The frequencies of red-periderm and white-periderm individuals in progenies of 11 various selfed parents are recorded in table 1. The distribution suggests a relation of 3:1 in family no. 85, the progeny of a plant of unknown tuber color, and 9:7 in 3 families and 27:37 in 3 families, progenies of selfed seedlings which had red tuber color only in periderm of the eye region. These numerical relationships would be expected if three interacting factors were operating to produce red color in the periderm and if the respective parents were segregating for 1, 2, and 3 factor pairs.

The relation of 45:19 suggested by the frequencies in progenies of 3 red-periderm parents, the Triumph variety and seedlings nos. 5-10-1 and 5-14-8-1, would be expected if these parents were heterozygous for 3 pairs of interacting factors, 2 of which were duplicates.

<sup>1</sup> Received for publication July 16, 1935; issued February, 1936. Journal Series Paper No. 1370 of the Minnesota Agricultural Experiment Station.

<sup>2</sup> Reference is made by number (italic) to Literature Cited, p. 63.

TABLE 1.—*Periderm color in progenies of certain selfed potato plants*

Seedling or variety no.	Periderm color of parent	How derived	Progeny having indicated periderm color			Suggested factorial formula	Theoretical ratio of red to white
			Red	White	Total		
			Number	Number	Number		
85.....	Unknown.....	{Observed.....	153	47	200	<i>EE SS Ff</i> .....	3.1
		{Calculated.....	150	50	200		
41-2-2-3.....	Red eye <sup>1</sup> .....	{Observed.....	125	95	220	<i>Ee Ss FF</i> .....	9.7
		{Calculated.....	123.75	96.25	220		
8-4.....	do. <sup>1</sup> .....	{Observed.....	94	89	183	<i>Ee Ss FF</i> .....	9.7
		{Calculated.....	103	80	183		
12-2-22.....	do. <sup>1</sup> .....	{Observed.....	75	43	118	<i>Ee Ss FF</i> .....	9.7
		{Calculated.....	66	52	118		
5-3.....	do. <sup>1</sup> .....	{Observed.....	213	252	465	<i>Ee Ss Ff</i> .....	27:37
		{Calculated.....	196	269	465		
5-6.....	do. <sup>1</sup> .....	{Observed.....	37	40	77	<i>Ee Ss Ff</i> .....	27:37
		{Calculated.....	32.5	44.5	77		
41-2-7.....	do. <sup>1</sup> .....	{Observed.....	30	56	86	<i>Ee Ss Ff</i> .....	27:37
		{Calculated.....	36	50	86		
5-10-1.....	Red.....	{Observed.....	223	89	312	<i>Ee Ss S's' FF</i> .....	45:19
		{Calculated.....	219.4	92.6	312		
5-14-8-1.....	do.....	{Observed.....	67	34	101	<i>Ee E'e' Ss FF</i> .....	45:19
		{Calculated.....	71	30	101		
Triumph.....	do.....	{Observed.....	252	102	354	<i>Ee E'e' Ss FF</i> .....	45:19
		{Calculated.....	249	105	354		
5-14.....	Light red.....	{Observed.....	35	26	61	<i>Ee E'e' Ss Ff</i> .....	135:121
		{Calculated.....	32	29	61		

<sup>1</sup> Red confined to periderm of eye region.

The proportion of red to red-eye periderm color was 71 to 152 in family no. 5-10-1, in contrast to the proportions of 198 to 54 and 49 to 18 in progenies of the Triumph and no. 5-14-8-1. This difference suggests that the pair of duplicate factors segregating in seedling 5-10-1 is not the same as the pair of duplicate factors heterozygous in the Triumph and in no. 5-14-8-1.

Its light red-periderm color, the distribution of color in its progeny, and in the progeny of its second-generation seedling no. 5-14-8-1 are considerations which suggest that seedling no. 5-14 was heterozygous for 4 pairs of interacting factors, 2 of which were duplicates.

### INHERITANCE OF CORTEX COLOR

Cortex color is assumed to be governed by four pairs of interacting genes. According to this working hypothesis, red-cortex color is considered to be due to the complementary action of factors *R*, *D*, and *C*, and blue cortex to result from the addition of a factor *P*.

### INHERITANCE OF RED COLOR IN THE CORTEX

As recorded in table 2, the proportions of red-cortex individuals in families resulting from selfing the Flourball and Early Ohio varieties and in the progeny, no. 10.22, of a cross between the Early Ohio and a white-cortex seedling suggest that these varieties were heterozygous for two interacting factors for cortex color. A similar condition is indicated for Early Ohio seedling no. 41-17 by its color and by the proportion of red-cortex individuals in family no. 11.

When five cortex-color seedlings of family 11 were selfed, the behavior of faint-red cortex seedlings nos. 11-4 and 11-8 indicated that they may have been heterozygous for three pairs of interacting

cortex color factors. The behavior of seedling no. 11-4 in crosses recorded in tables 2 and 3 supports this conclusion, especially when considered in comparison with the behavior of light-red cortex seedling no. 11-3 in similar crosses.

TABLE 2.—Cortex color in progenies of selfed red-cortex individuals and in progenies of certain red-cortex  $\times$  white-cortex crosses

Family no.	Parents and color	Progeny having indicated cortex color				Suggested factorial formula	Theoretical ratio of red to white
		How derived	Red	White	Total		
			<i>Number</i>	<i>Number</i>	<i>Number</i>		
7	Flourball (light red).....	{Observed.....	84	66	150	<i>CC Rr Dd</i> .....	9:7
		{Calculated.....	84	66	150		
41	Early Ohio (light red).....	{Observed.....	315	223	538	<i>CC Rr Dd</i> .....	9:7
		{Calculated.....	303	235	538		
10 22	{Early Ohio (light red) $\times$ no. 4.....	{Observed.....	17	39	56	<i>CC Rr Dd</i> $\times$ <i>cc Rr dd</i> .....	3 5
		{Calculated.....	21	35	56		
11	{No. 41-17 (light red) $\times$ no. 4-9-1 (white).....	{Observed.....	124	225	349	<i>CC Rr Dd</i> $\times$ <i>cc Rr dd</i> .....	3.5
		{Calculated.....	131	218	349		
	Selfed seedlings of family no. 11:						
11-4	No. 11-4 (faint red).....	-----	17	28	45	<i>Cc Rr Dd</i> .....	27 37
11-8	No. 11-8 (faint red).....	-----	7	9	16	<i>Cc Rr Dd</i> .....	27 37
11-3	No. 11-3 (light red).....	-----	26	10	36	<i>Cc RR Dd</i> .....	9 7
11-6	No. 11-6 (light red).....	-----	18	13	31	<i>Cc RR Dd</i> .....	9 7
11-7	No. 11-7 (light red).....	-----	31	15	46	<i>Cc RR Dd</i> .....	9 7
17 26	{No. 11-4 (faint red) $\times$ no. 4-27 (white).....	-----	7	11	18	-----	
22 26	{No. 11-3 (light red) $\times$ no. 4-27 (white).....	-----	8	38	46	-----	

TABLE 3.—Data relating to progeny inheritance of blue cortex color

Family no.	Parents and color	Progeny having indicated cortex color			
		Blue	Red	White	Total
		<i>Num-ber</i>	<i>Num-ber</i>	<i>Num-ber</i>	<i>Num-ber</i>
54 26	Russet Rural (white) $\times$ no. 11-4 (faint red).....	16	21	53	90
38 26	Russet Rural (white) $\times$ no. 11-3 (light red).....	42	52	108	202
53 26	Russet Rural (white) $\times$ no. 41-1 (white).....	0	0	31	31
40 26	41-1 (white) $\times$ no. 11-3 (light red).....	0	22	26	48

### INHERITANCE OF BLUE COLOR IN THE CORTEX

Data relating to the inheritance of blue-cortex color is recorded in table 3. Blue-, red-, and white-cortex seedlings were observed in families resulting from crosses between the white-cortex Russet Rural variety and red-cortex seedlings nos. 11-3 and 11-4. The nearly equal occurrence of blue- and red-cortex individuals in each of these families suggests that the Russet Rural may have been heterozygous for a factor for blue-cortex color and that this factor, designated *P*, when present with the factors necessary for red-cortex color, produced blue-cortex color. The behavior of white-cortex seedling no. 41-1, a seedling of the Early Ohio, in crosses with seedling 11-3 and Russet Rural suggests that seedling 41-1 and Russet Rural were probably recessive for the same factor pair.



## RELATION OF COLOR IN PERIDERM AND CORTEX

The individuals of family 85, progeny of a plant of unknown tuber color, are classified in table 4 in four color classes. The frequencies suggest a relation of 3:1 for periderm color and 9:7 for cortex color. A comparison of the observed frequencies with the combined ratio of 27:21:9:7 for the two character pairs show good agreement,  $P$  equaling 0.54. It appears that in family no. 85 a factor for periderm color is segregating independently of two factors for cortex color.

TABLE 4.—Segregation of periderm and cortex color in family no. 85

Item	Progeny having indicated color				
	Red periderm, red cortex	Red periderm, white cortex	White periderm, red cortex	White periderm, white cortex	Total
Observed.....	88	65	31	16	200
Calculated.....	84	66	28	22	200

The absence of blue periderm-red cortex and red periderm-blue cortex individuals in family no. 100.27, progeny of a cross between a blue-periderm and a red-cortex parent, as recorded in table 5, suggests that the factor  $P$  may operate in both periderm and cortex to change red color to blue. This view is in harmony with the data relating to progenies of crosses between the *Rural*, a smooth white tuber variety, and three selected parents, as recorded in table 6. Color was confined to the periderm in blue and red individuals in family no. 50.31 whose pollen parent was a red-periderm seedling. Red and blue color characterized only the cortex in those families whose pollen parents were derived from red-cortex parents. The 3:1 relation of blue to red apparent in each of these progenies suggests that the *Rural* may have been heterozygous for two duplicate factors,  $P$  and  $P'$ , each capable of changing red periderm and red cortex to blue.

TABLE 5.—Color classes in a cross of blue periderm  $\times$  red cortex (accession no. 125  $\times$  11-3-6), family no. 100.27

Cortex color	Progeny having indicated periderm color			Total
	Blue	Red	White	
Blue.....	Number 5	Number 0	Number 4	Number 9
Red.....	0	3	1	4
White.....	9	1	3	13
Total.....	14	4	8	26

TABLE 6.—Color in periderm and cortex in progenies of crosses between the Rural variety and certain pollen parents

Family no.	Parents and color	Progeny having color in—					
		Periderm		Cortex			Total
		Blue	Red	Blue	Red	White	
50 31	Rural (white) × no. 5-3-2 (red periderm)....	Number 20	Number 3	Number 0	Number 0	Number 30	Number 53
56 31	Rural (white) × no. 7-1-2 (white).....	0	0	26	9	26	61
58 31	Rural (white) × no. 11-4-16-2 (white).....	0	0	31	14	26	71

## SUMMARY

This paper is concerned with the inheritance of color in periderm and cortex of the potato, *Solanum tuberosum* L.

The segregation of red and white periderm color in progenies of 11 selfed potato plants has been explained by assuming that the complementary action of factors *E*, *S*, and *F* produced red color in the periderm and that certain parents carried duplicate factors.

The inheritance of red cortex color has been explained by assuming that the complementary action of factors *C*, *R*, and *D* produced red color in the cortex.

In one family a factor for red periderm color was segregating independently of two factors for red cortex color.

Blue color in periderm and cortex has been referred to two factors, *P* and *P'*, each capable of changing red periderm and red cortex to blue.

The observations suggest that while certain factors affect color in only one tissue other factors influence color in both periderm and cortex.

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# THE EFFECT OF CALCIUM CARBONATE AND SODIUM BICARBONATE ON THE TOXICITY OF GOSSYPOL<sup>1</sup>

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## INTRODUCTION

In a previous study of the influence of certain dietary constituents on the response of rats to gossypol ingestion (2)<sup>2</sup> the authors showed that the toxicity of diets containing known amounts of gossypol was materially reduced when the diets were made basic by the addition of calcium carbonate and sodium bicarbonate. When the diets were made acidic by the addition of calcium chloride, only indirect evidence of a slight decrease in toxicity was obtained. Decreased toxicity was observed also when the protein content of the diet was increased from 13 percent to 35 percent. The conclusion was drawn that diets of high protein content and basic diets of high calcium content are favorable to the detoxication of gossypol.

Considerable importance is attached to the results since calcium and protein are variable ingredients in diets used for the bio-assay of gossypol. The importance of these constituents in feeding cottonseed products to livestock has not been fully determined, although the value of supplementary protein in cottonseed-meal rations for pigs has recently been pointed out by Robison (5).

In view of the favorable results obtained with sodium bicarbonate and calcium carbonate, it was deemed desirable to determine the proportion of these salts which would offer the greatest degree of protection against gossypol injury and to determine the value of each salt in the presence of moderate amounts of the other, as the principal elements of both are requisite for a normal nutritive condition. The present paper reports the results of this study. The results of other experiments, in which calcium altered the inhibiting effect of gossypol on the hydrolysis of fat by lipase, are also reported. The results of these studies are offered as further evidence of the important part played by calcium in the detoxication of gossypol.

## MATERIAL AND METHODS

The manner of conducting the feeding experiments, in which albino rats were used as experimental animals, has been described in a previous paper (2).

The percentage composition of the basal diet to which varying amounts of sodium bicarbonate and calcium carbonate were added was as follows: Cottonseed 10, starch 49, casein 24, yeast 5, cod-liver oil 3.5, salt mixture (3) 3.5, and Crisco 5. The cottonseed contained 0.60 percent of gossypol. Control diets were made up with gossypol-free

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<sup>2</sup> Reference is made by number (italic) to Literature Cited, p. 72.

cottonseed. Fresh diets were prepared weekly, at which time the prescribed amounts of calcium carbonate and sodium bicarbonate were added. In one experiment sodium bicarbonate was added to the basal diet at the time of feeding, and any food residues from the previous day were discarded. This procedure was followed as a precaution against the possible destruction of the gossypol, by contact with the alkali, before ingestion.

In the lipase studies the general procedure of Willstätter, Waldschmidt-Leitz, and Memmen (7) was followed. A glycerol extract of pig's pancreas was used as a source of lipase. The fresh tissue was dried with acetone and ether and ground in a mortar with 16 times its weight of 80-percent glycerol. After standing at room temperature for 4 hours or longer, with occasional shaking, the solution was filtered through filter paper and stored in an electric refrigerator.

Neutral olive oil was prepared by shaking a high-grade commercial oil with an equal volume of 1-percent sodium hydroxide in a separatory funnel at 60° C. After the aqueous layer had been removed, the oil was thoroughly washed with hot water and dried in the electric oven at 100° C. for 6 hours.

A 2-percent solution of egg albumin was prepared by dissolving the dry powder in an excess of ammonium hydroxide and, after this had been shaken for several hours, removing the excess ammonia with a current of warm air.

Two buffer solutions were prepared. One was composed of 66 parts of N/1 ammonium hydroxide and 134 parts of N/1 ammonium chloride and had a pH of 8.9 at 30° C. The other buffer solution was composed of equal parts of N/2 acetic acid and N/2 sodium acetate and had a pH of 4.7 at 30°.

A 1-percent solution of calcium chloride was made up with the chemically pure salt. Gossypol was prepared and purified according to methods described by Clark (1).

Determinations of the rate of hydrolysis of olive oil by lipase in the presence of calcium and gossypol were made in the following manner (4). One-half cubic centimeter of lipase extract, 3 cubic centimeters of olive oil containing various amounts of gossypol, 2 cubic centimeters of the alkaline buffer solution, 1 cubic centimeter of calcium chloride solution, and sufficient amounts of a dilute glycerol solution to keep the total volume of the water 11 cubic centimeters and the total volume of glycerol 2 cubic centimeters, were shaken together in a small Erlenmeyer flask. One cubic centimeter of egg-albumin solution was then added, and after being shaken for 3 minutes, the flask was placed in a constant-temperature water bath held at 30° C  $\pm$  0.2 for 2 hours and 57 minutes. At the end of this time the contents of the flask were washed into a 250-cubic centimeter Erlenmeyer flask with 112 cubic centimeters of 95-percent alcohol. Twenty cubic centimeters of ether was added and the fatty acids titrated with N/10 alcoholic sodium hydroxide, thymolphthalein being used as an indicator. Blank determinations were carried out without the addition of the enzyme.

Determinations in which calcium and albumin were omitted were made in the manner described above. In another series of experiments the gossypol was dissolved in the buffer instead of in the oil. In a final series of experiments hydrolysis was carried out in an acid medium with 2 cubic centimeters of the acid buffer solution replacing the alkaline buffer solution. The gossypol was dissolved in the oil.

## EXPERIMENTAL RESULTS

## FEEDING EXPERIMENTS WITH CALCIUM CARBONATE AND SODIUM BICARBONATE

Data obtained with rats which received diets containing 0.06 percent of gossypol and varying amounts of calcium carbonate and sodium bicarbonate, together with their controls, are shown in table 1.

Table 1 shows a conspicuous difference between the food intake of animals receiving gossypol and the intake of their controls. That of the rats on gossypol was noticeably increased by the addition of sodium bicarbonate to the diet. The same result was obtained with combinations of this salt with calcium carbonate, although the latter by itself tended to decrease the food intake of rats on gossypol diets. The average food intake of the gossypol rats on diets containing 3 percent of sodium bicarbonate slightly exceeded that of their controls, being 599 grams as compared with 585 grams. Similarly there was very little difference between the food intake of the controls and that of the gossypol rats on a diet to which a combination of 2 percent of sodium bicarbonate and 2 percent of calcium carbonate had been added. The increase in food consumption on the part of the rats on gossypol is taken as an indication of improvement in the nutritive condition of the animals.

The average gains in weight of rats on the gossypol diets likewise increased with increasing percentages of sodium bicarbonate in the diet. At the 3-percent level, sodium bicarbonate was particularly effective in producing an increase in their gains. The greatest average gain for the rats on gossypol, 118 grams, was made on a diet containing 2 percent of calcium carbonate and 2 percent of sodium bicarbonate, although the average gains made by gossypol rats on diets containing 3 percent of sodium bicarbonate or a combination of 2 percent of calcium carbonate and 1 percent of sodium bicarbonate approached this value.

TABLE 1.—*Effect of gossypol on growth of rats as influenced by the addition of various amounts of sodium bicarbonate and calcium carbonate to the diet*

Salt additions to basal diet	Gossypol content of diet	Rats	Average food consumed in 60 days <sup>1</sup>	Average gain in weight in 60 days <sup>1</sup>	Difference in gains in weight
	Percent	Number	Grams	Grams	Grams
None.....	0.06	12	468	88	82
	.00	7	629	170	
1 percent of NaHCO <sub>3</sub> .....	.06	14	530	92	71
	.00	6	703	163	
2 percent of NaHCO <sub>3</sub> .....	.06	12	527	99	50
	.00	6	659	149	
2 percent of NaHCO <sub>3</sub> 2 percent of CaCO <sub>3</sub> <sup>2</sup> .....	.06	6	530	94	55
	.00	6	659	149	
3 percent of NaHCO <sub>3</sub> .....	.06	7	599	106	44
	.00	7	585	150	
2 percent of CaCO <sub>3</sub> .....	.06	6	413	87	76
	.00	6	678	163	
2 percent of CaCO <sub>3</sub> ; 2 percent of NaHCO <sub>3</sub> .....	.06	7	624	118	28
	.00	6	630	146	
2 percent of CaCO <sub>3</sub> ; 1 percent of NaHCO <sub>3</sub> .....	.06	9	500	102	58
	.00	9	621	160	

<sup>1</sup> The sum of the averages obtained with males and females divided by 2.

<sup>2</sup> 2 percent of sodium bicarbonate added daily to gossypol diet. The figures presented for the controls in this group are those given for the controls in the preceding group.

An important point brought out in table 1 is that the addition of 2 percent of calcium carbonate by itself effected no improvement in

the growth of the gossypol or control rats; its addition in combination with 1 percent or 2 percent of sodium bicarbonate produced a noticeable improvement in the gains made by the gossypol rats, increasing their average gain from 88 grams to 102 and 118 grams. This combination of salts was likewise more effective than single additions of 1 percent or 2 percent of sodium bicarbonate.

It should be noted that in the case of the control rats the addition of sodium bicarbonate, especially in amounts exceeding 1 percent, either alone or in combination with calcium, was unfavorable to gains in weight and in one instance (3 percent of sodium bicarbonate) caused decreased food consumption. Since the opposite effect was produced in the case of the rats on gossypol, the difference between gains made by control and gossypol rats on diets containing over 1 percent of sodium bicarbonate becomes small, being 55 grams or less, as compared with 82 grams. One criterion used to judge the effectiveness of each dietary alteration is that the effect so produced be more apparent in the performance of the rats on gossypol than in their controls. It seems reasonable therefore to believe that by the addition of such a readily soluble base as sodium bicarbonate conditions are produced that are favorable to the detoxication of gossypol although unfavorable to the growth of animals on a normal diet.

In order to facilitate a comparison of the gains made by gossypol rats on what appeared to be the least effective combinations of salts with those that received the most effective combinations, the animals have been divided into three groups. Group 1 includes the rats which, with respect to salt additions, received the following three diets: No addition, 1 percent of sodium bicarbonate and 2 percent of calcium carbonate. These made average gains between 87 and 92 grams. Group 2 consists of those which received 2 percent of sodium bicarbonate. In group 3 are those which received 3 percent of sodium bicarbonate and the two combinations of sodium bicarbonate and calcium carbonate. These made average gains between 102 and 118 grams. The average gain in weight made by the rats that received 2 percent of sodium bicarbonate falls between the average gains in weight of the rats in the first and third groups. The average gains and standard deviations of the means for males and females in these three groups are given in table 2. The formula used for the standard deviation was

$$\sigma = \sqrt{\frac{\sum d^2}{N}}$$

in which

$\sigma$ =standard deviation of the average,  
 $\sum d^2$ =sum of the deviations from the average, squared, and  
 $N$ =number of observations.

Table 2 shows the beneficial effect of increased amounts of calcium carbonate and sodium bicarbonate in diets containing gossypol. Although it is not possible to make a sharp distinction between the different amounts and combinations of these salts with respect to their detoxicating properties, the amounts added to the diets of group 3 were undoubtedly more effective than the single additions to the diets of group 1. This is more apparent in the average growth of females than of males. That this result was not due to the increased

nutritive value of the diets is shown by the performance of the control rats.

TABLE 2—*Gains in weight made by 3 groups of rats on gossypol and gossypol-free diets*

Sex of rats and groups <sup>1</sup> compared	60-day gains made by rats on—									
	Gossypol diets					Gossypol-free diets				
	Rats	Minimum gain	Maximum gain	Average gain	Standard deviation	Rats	Minimum gain	Maximum gain	Average gain	Standard deviation
Males:	<i>Number</i>	<i>Grams</i>	<i>Grams</i>	<i>Grams</i>	<i>Grams</i>	<i>Number</i>	<i>Grams</i>	<i>Grams</i>	<i>Grams</i>	<i>Grams</i>
Group 1.....	18	75	141	109	19.92	7	180	217	202	11.09
Group 2.....	10	77	166	108	27.00	3	169	182	175	-----
Group 3.....	15	77	167	125	28.78	13	155	217	186	17.61
Females										
Group 1.....	12	41	115	72	17.55	12	102	153	129	13.71
Group 2.....	8	63	114	87	15.26	3	100	137	123	-----
Group 3.....	8	69	108	91	13.27	9	112	125	120	4.58

<sup>1</sup> Group 1 includes rats which, with respect to salt additions, received the following 3 diets: No addition, 1 percent of  $\text{NaHCO}_3$ , and 2 percent of  $\text{CaCO}_3$ ; group 2 includes rats which received 2 percent of  $\text{NaHCO}_3$ , group 3 includes rats which, with respect to salt additions, received the following 3 diets: 3 percent of  $\text{NaHCO}_3$ , 2 percent of  $\text{CaCO}_3$ , +2 percent of  $\text{NaHCO}_3$ , and 2 percent of  $\text{CaCO}_3$ , +1 percent of  $\text{NaHCO}_3$ .

#### LIPASE STUDIES

The effect of increasing amounts of gossypol on the hydrolysis of fat by lipase, both alone and in combination with calcium and albumin is shown in table 3. In experiment 25, which was carried out in a system with an initial pH of 8.9, the inhibition of hydrolysis produced by 8 milligrams of gossypol dissolved in the oil amounted to 63 percent. In the presence of calcium, a substance which normally activates the reaction, hydrolysis was practically stopped by 8 milligrams of gossypol. The same result was obtained when both activators, calcium and albumin, were present.

In experiment 26, in which another enzyme preparation was used gossypol was dissolved in the buffer solution. In the absence of activators, a maximum inhibition of 58.6 percent was produced by 8 milligrams of gossypol. With albumin as an activator, the amount of inhibition was slightly increased by 12 milligrams of gossypol. In the presence of calcium, however, this amount of gossypol practically stopped hydrolysis.

Experiment 28 was conducted in a system with an initial pH of 4.7. This was secured by replacing the alkaline buffer solution with an acetate buffer solution. In this system, albumin inhibited the hydrolysis, and calcium was without appreciable effect. The amount of inhibition produced by 12 milligrams of gossypol dissolved in the oil was approximately the same as that in experiment 25, being 71.8 percent. Gossypol in the presence of calcium did not completely prevent hydrolysis, as it did in the presence of the alkaline medium.

The correct interpretation of these results based upon proven facts must necessarily await more complete knowledge of the action of both lipase and gossypol. Three important facts have been established: (1) Gossypol inhibits lipase activity in basic and acidic media; (2) complete inhibition by gossypol in an alkaline medium is dependent upon the presence of calcium, and in an acid medium the degree of



inhibition is independent of the calcium; (3) calcium precipitates gossypol from an alkaline solution (2).

TABLE 3—Effect of gossypol on lipase action both alone and in combination with calcium and albumin in basic and acidic media as shown in 3 experiments

[Period of hydrolysis, 3 hours; temperature, 30° C]

EXPERIMENT 25, ENZYME 4; GOSSYPOL DISSOLVED IN OIL, INITIAL pH, 8.9

Albu- min (milli- grams)	Cal- cium chlo- ride	Gossy- pol	Hydro- lysis in terms of N/10 NaOH used in titra- tion	Inhibi- tion in terms of N/10 NaOH used in titra- tion	Inhibi- tion	Albu- min (milli- grams)	Cal- cium chlo- ride	Gossy- pol	Hydro- lysis in terms of N/10 NaOH used in titra- tion	Inhibi- tion in terms of N/10 NaOH used in titra- tion	Inhibi- tion
	Milli- grams	Milli- grams	Cubic centi- meters	Cubic centi- meters	Percent		Milli- grams	Milli- grams	Cubic centi- meters	Cubic centi- meters	Percent
0-----	0	<div><div>0</div><div>4</div><div>8</div><div>12</div></div>	<div><div>7.3</div><div>3.9</div><div>2.7</div><div>2.1</div></div>	<div><div></div><div>3.4</div><div>4.6</div><div>5.2</div></div>	<div><div></div><div>46.6</div><div>63.0</div><div>71.0</div></div>	20-----	10	<div><div>0</div><div>4</div><div>6</div><div>8</div><div>10</div><div>12</div></div>	<div><div>21.4</div><div>15.5</div><div>1.2</div><div>.8</div><div>.8</div><div>.8</div></div>	<div><div></div><div>5.9</div><div>20.2</div><div>20.6</div><div>20.6</div><div>20.6</div></div>	<div><div></div><div>27.6</div><div>94.4</div><div>96.3</div><div>96.3</div><div>96.3</div></div>
0-----	10	<div><div>0</div><div>4</div><div>8</div><div>12</div></div>	<div><div>20.0</div><div>10.3</div><div>1.0</div><div>6</div></div>	<div><div></div><div>9.7</div><div>19.0</div><div>19.4</div></div>	<div><div></div><div>48.5</div><div>95.0</div><div>97.0</div></div>						

EXPERIMENT 26; ENZYME 5, GOSSYPOL DISSOLVED IN BUFFER; INITIAL pH, 8.9

0-----	0	0	7.0	0	0	0-----	10	0	25.0	5.9	22.4
		2	7.0	0	0			2	19.4	5.6	51.2
		4	5.0	2.0	28.6			4	12.2	12.8	82.4
		8	2.9	4.1	58.6			8	4.4	20.6	96.8
		12	2.9	4.1	58.6			12	.8	24.2	
20-----	0	0	15.0								
		2	13.1	1.9	12.7						
		4	11.3	3.7	24.7						
		8	5.8	9.2	61.3						
		12	5.0	10.0	66.6						

EXPERIMENT 28, ENZYME 5, GOSSYPOL DISSOLVED IN OIL; INITIAL pH, 4.7

0-----	0	0	8.5	4.8	56.5	0-----	10	0	8.6	4.7	54.7
		2	3.7	5.3	62.4			2	3.9	5.4	62.8
		4	3.2	5.5	64.7			4	3.2	6.2	72.1
		8	3.0	6.1	71.8			8	2.4	6.4	74.4
		12	2.4					12	2.2		
20-----	0	0	2.5								
		2	1.7	.8	32.0						
		4	1.5	1.0	40.0						
		8	1.4	1.1	44.0						
		12	1.2	1.3	52.0						

Waldschmidt-Leitz (6) has explained the activating effect of calcium on lipolysis as being due to "the production of colloid particles which exert an absorbent action with respect to the enzyme as well as to the substrate and thereby facilitate the reaction." Gossypol, by its affinity for calcium in alkaline solution, apparently prevents the formation of this "coupled absorbent"<sup>3</sup> or, as seems more likely, completely removes it from its sphere of activity.

The relationship of these observations to the results of the feeding experiments is discussed later.

<sup>3</sup> A "coupled absorbent" involving calcium and albumin has been represented by Willstätter et al. (7, p. 100) as calcium oleate—albumin  
fat lipase

## DISCUSSION

The foregoing feeding experiments have demonstrated the beneficial effect of sodium bicarbonate and calcium carbonate on the growth of rats receiving toxic diets which contained 0.06 percent of gossypol. The increased growth of the rats which received 3 percent of sodium bicarbonate or combinations of 1 and 2 percent of sodium bicarbonate with 2 percent of calcium carbonate was shown to be due to the action of these salts on gossypol since no corresponding increase in growth rate was produced by the addition of these salts to a gossypol-free diet of the same composition. In fact sodium bicarbonate in amounts exceeding 1 percent proved detrimental to the growth of rats on gossypol-free diets. That the beneficial effect of these salt additions was not due merely to an increase in potential alkalinity of the diets is shown in the poor performance of the animals that received 2 percent of calcium carbonate. In other experiments (unpublished) 3 percent of calcium carbonate has likewise been found to be ineffective.

The value of 3 percent of sodium bicarbonate in gossypol diets is attributed to the solubility of this salt with the production of an alkaline medium in which gossypol is very reactive and unstable. Conditions are thereby made favorable for the reaction of gossypol with calcium and possibly other constituents normally present in the diet (2). Iron salts are particularly efficacious detoxicants.

The inability of calcium salts to detoxicate gossypol in the absence of sodium bicarbonate was in all probability due to an unfavorable reaction medium. When dissolved in dilute alkali, gossypol is slowly precipitated by calcium salts.

The activity of gossypol in an alkaline medium and its probable reaction with calcium was again indicated in a study of the effect of gossypol on the hydrolysis of fat by lipase. Gossypol inhibited the hydrolysis in both acidic and basic media. In an acid medium the degree of inhibition produced by gossypol was unaffected by the presence of calcium. In an alkaline medium, inhibition by gossypol was altered by calcium; and, despite the activating action of calcium, hydrolysis practically stopped under these conditions. Apparently a reaction took place between calcium and gossypol which involved the enzyme. A reaction between gossypol and the activator-enzyme complex of which calcium is a part seems probable.

## SUMMARY

The effect of calcium carbonate and sodium bicarbonate on the toxicity of gossypol diets was determined by feeding experiments with young rats. The experimental diets were made up with 10 percent of cottonseed which contained 0.6 percent of gossypol. Calcium carbonate and sodium bicarbonate were added to the diets in varying proportions and amounts. Similar additions were made to control diets made up with gossypol-free cottonseed. The criterion used to judge the effectiveness of these salt additions in modifying the deleterious action of gossypol was that the effect so produced be more apparent in the growth of the rats on gossypol diets than in that of their controls.

The best growth of the rats receiving gossypol was obtained with a diet to which 2 percent of calcium carbonate and 2 percent of sodium bicarbonate had been added. Next in value were diets to which 3 percent of sodium bicarbonate or a combination of 2 percent of calcium carbonate and 1 percent of sodium bicarbonate had been added. It was evident from the performance of the control rats that the beneficial effect of these salts was due to their action on gossypol and not merely to their influence on the nutritive properties of the basal diet.

The value of sodium bicarbonate in conjunction with calcium carbonate or in the presence of normal amounts of dietary calcium, was attributed to its solubility with the production of an alkaline medium in which gossypol is unstable and susceptible of precipitation by calcium. Support for this belief was found in a study of the effect of gossypol on the hydrolysis of fat by lipase, a process which when initiated in an alkaline medium, is activated by calcium.

In a system with an initial pH of 4.7 (acid region) 12 milligrams of gossypol inhibited but failed to prevent hydrolysis. This result was obtained both in the presence of calcium and in its absence. In a similar system with an initial pH of 8.9 (alkaline region) gossypol, in the absence of calcium, only inhibited hydrolysis; whereas in the presence of calcium, 8 milligrams of gossypol almost entirely prevented hydrolysis.

The conclusion is drawn that the action of calcium carbonate and sodium bicarbonate in gossypol diets is one of detoxication, involving a reaction between gossypol and calcium in an alkaline medium.

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# APPARATUS AND PROCEDURE FOR SEPARATING COTTON ROOT ROT SCLEROTIA FROM SOIL SAMPLES <sup>1</sup>

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## INTRODUCTION

In field studies of rotation, cultural, and soil-amendment practices in relation to the control of the cotton root rot disease, caused by *Phymatotrichum omnivorum* (Shear) Duggar, the number or percentage of dead and dying plants and crop yields has been used as a measure of the effectiveness of the treatment. How the causal organism is affected by such practices has received little or no attention.

The disease is perpetuated by sclerotia or resting bodies and infected roots with superficial or closely associated mycelial strands. In clean fallows and fields planted to nonsusceptible crops, if no infected roots of susceptible plants are present, sclerotia are the only means of carry-over. In the black Houston clay soils and other soil types of central Texas the sclerotia are brown in color when mature and vary from less than a millimeter to a centimeter or more in size. The bulk of sclerotia are found at soil depths of between 8 and 36 inches. Inasmuch as sclerotia may be present in large quantities and may remain viable for a number of years, it is important to know how any control treatment affects their viability and dissipation.

To study sclerotia in the laboratory it is necessary to separate them from the soil. Because of their small size it is impractical to separate by hand during a season or part of a season sufficient samples to be of much value. Washing in screen flats with a garden hose, as reported by various workers <sup>2 3 4</sup> is a method applicable only to pot or other small samples. By means of the apparatus and methods here described, from 10 to 15 barrel samples of soil, weighing from 2 to 4 tons may be handled, and the sclerotial separation completed in a single day.

## DESCRIPTION OF APPARATUS AND WASHING PROCEDURE

The machine used in the initial separation of sclerotia and soil is shown in figure 1. The essential parts of the machine are the frame, the cradlelike hoisting or tilting and feeding device, a vertically mounted, coarse-mesh cylinder screen with agitator, a fine-mesh horizontal screen below the cylindrical screen, and a splash-drain vat below and surrounding the lower screen.

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The frame proper is made of  $\frac{3}{8}$  by 2 by 2 inch angle iron, braced with  $\frac{3}{8}$ -inch steel plate. All the joints are welded. At the base, the machine is 32 by 32 inches, tapering to a smaller dimension at the top.

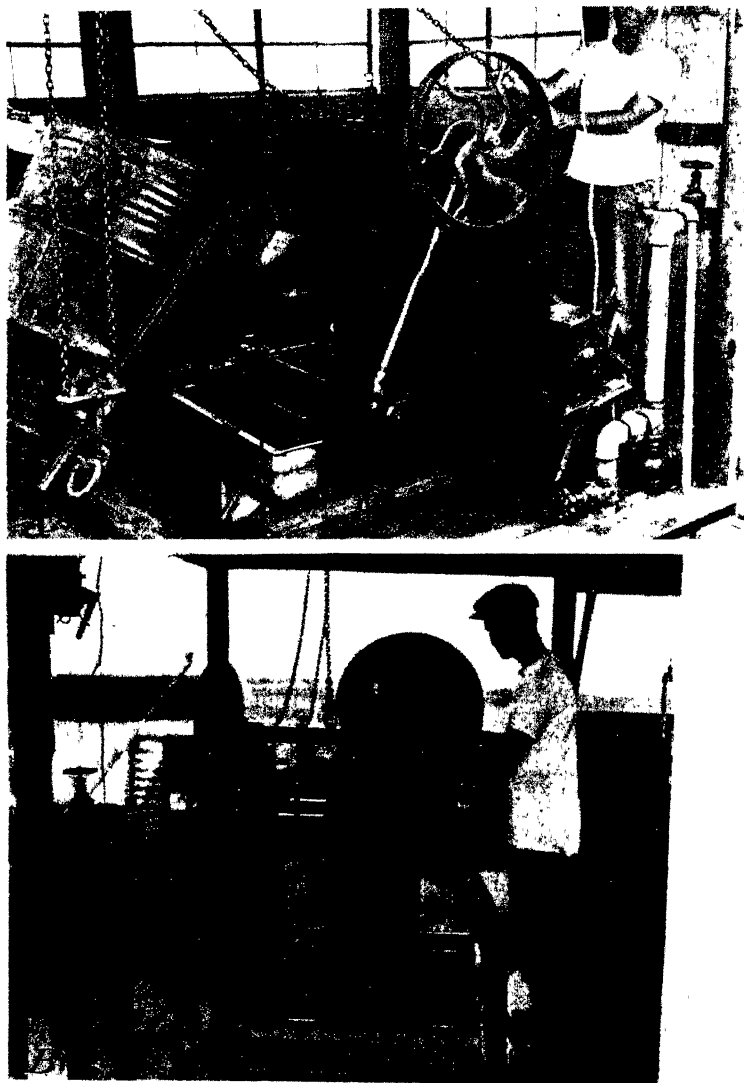


FIGURE 1.—Machine used in separating root rot sclerotia from soil: A, Side view, showing barrel-hoisting and tilting-frame motor, eccentric shaft (a), and adjustable overflow (b); B, front view of machine in operation. The soil from the barrel is washed directly into the cylinder screen.

It is approximately 40 inches high. A bowllike chute, made of  $\frac{1}{4}$ -inch plate, is welded around the top of the machine and tapers downward to the upper edge of the vertical screen cylinder. It is made wide enough for samples to be poured in or washed from a barrel

without any waste. The machine is driven by a small electric motor (one-third horsepower), seated on a swinging base. The large pulley used on the main drive shaft reduces its speed to approximately 250 revolutions per minute. The motor and belt are protected from splashing water by a panel of 18-gage iron bolted to the side of the frame adjacent to the motor. The main shaft and the eccentric and agitator shafts, mentioned later, are equipped with ball bearings, lubricated through grease cups.

Inasmuch as barrel-size samples are usually used in this work, a hoisting and tilting frame is attached to facilitate handling. It is made of the same material as the frame of the machine, and is constructed of such a height that the upper edge of the barrel extends about 6 inches over the chute around the top of the vertical screen. The top of the frame is hinged from the edge of the chute. A half-ton chain hoist attached to a rafter provides for the necessary lifting power. Oil drums of uniform height are used for sample containers.

Because of the puddling and the highly colloidal nature of the Houston soils, it is necessary to use a rather large quantity of water and thorough agitation to screen the material rapidly. The sample is first washed through the coarse cylinder screen and drops onto a finer screen below.

This cylinder screen is 12 inches in diameter, and 15 inches high, and is made of 8 by 8 mesh (8 meshes per linear inch) no. 16 (Washburn & Moen gage) iron wire. The openings and wire are each one-sixteenth of an inch in diameter. It is necessary to use a heavy wire, because of the harsh action of shell and pieces of plant stems or occasional small rocks that are present in samples of soil. The agitator in this screen cylinder is made of 1½-inch shafting with small rods inserted through the lower 15 inches of the shaft in a staggered manner. It is rigidly supported at the top with ball bearings and is operated from the pulley or main shaft by beveled gears. The size ratio of the two gears is such that the speed of the agitator is reduced to 150 r. p. m. Bearings and gears are enclosed to retain oil and protect them from silt and water.

To keep the sample in suspension as much as possible while agitation and screening are taking place, the water is supplied through numerous vents in a removable compartment at the bottom of this screen. This water-inlet compartment is shown partially removed in figure 2, *A*. It is also made of ¾-inch steel plate, with vents one thirty-second of an inch in diameter spaced about 1 inch apart over the top surface. A connecting hose supplies water under enough pressure to eject streams upward 2 or 3 feet through the small vents. The inlet compartment is held snugly against the bottom of the cylinder by a grooved flange support. Since the greater number of the sclerotia pass through this screen, it is necessary to pass the material on to a finer screen.

The horizontal screen, upon which the washings fall, is 36 inches long, 24 inches wide, and 4 inches deep. It is made of 35-mesh no. 29 (Washburn & Moen gage) brass-wire cloth with openings of 0.0136 inch. No. 12 sheet steel is used for the framework for this wire. Screen surfaces are provided at each end of the screen as well as the bottom, but the sides are solid (fig. 2, *B*). A reciprocating motion is provided for the screen by an eccentric shaft (fig. 1, *A*, *a*).

The length of screen stroke is varied by changing the position of the attachment on the lower end of the eccentric shaft on the arm of the screen-propelling shaft. A stroke of 2 inches is most satisfactory.



FIGURE 2.—A, Close view of part of the machine showing cylinder screen, water inlet and hose, horizontal screen supporting shafts (*a, a*), and splash-drain vat (*b*); B, horizontal fine-mesh screen.

The screen is supported by the two shafts shown in figure 2, A, *a, a*, and is held in place by four pieces of U iron, welded on the bottom of the screen frame. To withstand the grinding action of silt and water, these pieces of U iron are lined with a hard steel alloy. The

areas on the shafts where these bearings rest are coated with the same material. To remove the screen, it is necessary only to lift it from these shafts. These two shafts are suspended by arms at each end from 4-inch plain bearings, each supplied with a grease cup. The rear shaft and arms hang loose, but the forward, or propelling, arms and shaft, are rigidly connected to the eccentric. A rubber hood is tied over each of the bearings to preserve them against silt and water.

To aid screening on the fine-mesh screen and facilitate proper drainage, a pyramid-shaped vat is supported around and below the screen. Water and silt from the vat are drained through a 2-inch drainpipe. While in operation, the water level in this vat is kept only about half an inch above the bottom of the screen by an adjustable overflow in the wall of the vat (fig. 1, *A*, *b*). By keeping the water at this low level, screening is not only accelerated, but splashing, caused by the movement of the screen, is reduced to a minimum. A valve in the drainpipe is controlled from above the floor by an extension handle, shown projecting just below the motor in figures 1, *A*, and 2, *A*. This valve is left partially open during screening to allow silt to move out freely. The vat is quickly filled or emptied by closing or opening the valve.

In screening a sample of soil, the sample is either emptied by hand or washed from a barrel into the cylinder screen. The finer particles, and most of the sclerotia, are washed through the cylinder onto the horizontal screen below. When screening is complete, the water inlet, at the base of the cylinder screen, is partly or entirely slid from its flange support and the residue dumped or washed onto the lower screen. The lower screen is then removed and the total residue of both screens washed into a suitable container. There is usually about half a gallon to a gallon of this residue left from a barrel of Houston soil. It is composed primarily of shell and bits of organic matter and any sclerotia that might be present.

#### RELATION OF RETENTION OF SCLEROTIA TO SIZE OF SCREEN

To determine just how fine a screen should be used in order to retain all sclerotia in screening procedures, a number of screens of different-size meshes have been used. The finest wire previously used was a 20-mesh per inch, similar to ordinary window screen. In a series of tests, an 18-mesh iron wire was used on the cylinder, a 40-mesh brass wire on the lower screen, and 60-mesh brass wire was placed under the outlets of the drain and overflow pipes. These tests showed that an average of 70 percent of the sclerotia passed the 18-mesh wire but that none passed the 40-mesh wire, as is shown by the fact that none was caught on the 60-mesh wire. The 18-mesh wire on the cylinder screen has since been changed to the heavier 8 by 8 mesh, and the wire on the horizontal screen has been changed from the 40-mesh to the 35-mesh. This 35-mesh screen is made of heavier wire than the 40-mesh and has approximately the same-size openings. Rea<sup>5</sup> found, in a number of tests, that 80 percent of the sclerotia passed a 16-mesh brass wire and 7 percent passed a 30-mesh wire. The openings in this 30-mesh wire were 0.021 inch and were considerably finer than any previously reported as being used for this work.

<sup>5</sup>REA, H. E. RECLAIMING COTTON ROOT ROT SCLEROTIA FROM SOIL SAMPLES. Tex. Agr. Expt. Sta., Substa. 5, Ann. Rept. 1933. [Not published.]



## SEPARATION OF SCLEROTIA FROM SCREEN RESIDUE

To further reduce the amount of material from which sclerotia must be picked, the residue is stirred with a sugar solution of specific gravity of 1.15 to 1.25, or about 110 to 150 parts of sugar to 100 parts of water. This allows the sclerotia, which are slightly heavier than water, to float, while the heavier shell and gravel sink. The sclerotia, along with other light organic matter, are then decanted off. To assure the removal of all the sclerotia, this procedure is repeated twice. A few other materials were tried for this purpose. Sugar was found to be the best, however, because it is not expensive, is not toxic to sclerotia, and is readily obtainable. The sugar solution is kept at the desired specific gravity by testing with a hydrometer and adding sugar when necessary. When sclerotia are to be sepa-

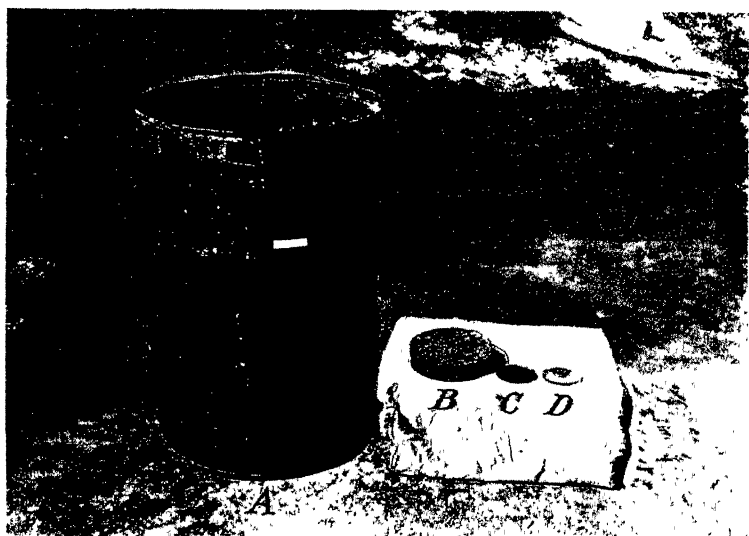


FIGURE 3.—Screen residue left after washing a barrel of soil (A), sclerotia (D) and rubbish (C) have been separated from shell and gravel (B) with sugar solution.

rated with the sugar solution, the material in the container is allowed to stand a few seconds after being stirred before the decanting is begun. The sclerotia and residue are then decanted onto a fine-mesh strainer and washed thoroughly under the tap to remove all sugar. In figure 3 are shown the fractions in the separation procedure. The three dishes at the right (B, C, D) contain the total amount of screen residue from the barrel of soil at the left. The two Petri dishes (C, D) contain the organic matter and sclerotia separated from the screen residue by using the sugar solution. The Petri dish (D) at the right contains the sclerotia (approximately 200) picked by hand with forceps from the organic matter in the other Petri dish.

A number of tests were made to determine whether the sugar used in this procedure had any effect on the germination of sclerotia. In these tests, quantities of sclerotia were picked from six different samples, after they had been screened and just before they were

treated with sugar, and compared with sclerotia separated from the same samples with the sugar solution. There was very little difference in the germination of the sclerotia separated by the two methods. The average germination of those removed by hand was 52 percent and that of those separated with the sugar solution, 54 percent.

#### SUMMARY

The sclerotial stage of the cotton root rot fungus *Phymatotrichum omnivorum* (Shear) Duggar is one of the most important means of perpetuating the root rot disease in Texas.

Sclerotia in the Houston clay soils of central Texas vary in size from less than a millimeter to a centimeter or more.

Apparatus and procedures for the rapid mechanical separation of sclerotia from soil samples are described.

In the initial machine separation, the soil is reduced to a finely divided state by the aid of water and a rotary agitator in a coarse-mesh cylinder screen. The material passes onto a finer screen partially submerged in water and moving in a reciprocating manner. As much as one-half gallon to a gallon of residue consisting of shell, gravel, and sclerotia remain in both screens when a barrel-size sample of soil is mechanically separated. The sclerotia are floated from the heavier shell and gravel residue by stirring in a sugar solution with a specific gravity of 1.15 to 1.25. The sugar solution does not affect the viability of the sclerotia.



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## MODIFICATION OF A MENDELIAN RATIO IN MAIZE BY POLLEN TREATMENTS<sup>1</sup>

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### INTRODUCTION

Of the millions of pollen grains produced by a single maize plant (*Zea mays* L.) not many more than 1 in 25,000 effect fertilization; the others perish. To a large extent chance determines which grains function and which are wasted, but survival or demise is not wholly a matter of chance. An element of selection intervenes, resulting in a differential survival value of certain sorts of pollen grains.

Some pollen grains bear within them a hereditary pattern that precludes functioning irrespective of external agencies. Others have a genetic complex that enables them to survive certain unfavorable environments. An example of the latter class is found in the so-called waxy gene—the hereditary element that produces a form of starch giving a red instead of a violet reaction with iodine. Pollen grains possessing the waxy gene, and in consequence having red-staining starch, sometimes survive conditions that are lethal to grains containing the form of starch normal to commercial varieties.

Some years ago, in the course of an investigation of the factors affecting variability of cross-over ratios in maize, it was unexpectedly discovered that the proportion of functioning pollen grains bearing the waxy gene to those bearing its dominant allelomorph could be profoundly altered by storing the pollen for several hours in the sunlight. Storage for the same length of time in the dark was ineffective.

This phenomenon was verified for 3 successive years, while attempts were made to standardize treatments and develop a technique that would result in a uniform reaction. Satisfied by these early experiments that the observed results were real, a project was launched in an attempt to determine the causal factors that operated during the storage period. The results were disconcerting in that the effects of many of the treatments contradicted the work of previous years.

Consequently a return was made to the original technique, with the surprising result that treatments formerly effective in increasing the proportion of functioning waxy<sup>2</sup> pollen grains were found now to be

<sup>1</sup> Received for publication Sept. 19, 1935, issued March 1936

<sup>2</sup> The two sorts of pollen grains dealt with in this study are designated "horny" or "waxy" depending upon which member of the allelomorphic pair *Wx*, *wx* they bear. No ambiguity should result from this practice, and by this means the awkward expressions "waxy bearing" and "nonwaxy bearing" are avoided while the advantage of contrasting names is obtained.

lethal. Pollen exposed to direct sunlight for periods even as short as 10 minutes was completely devitalized.

The accumulating inconsistencies, probably rather more than the merits of the problem itself, stimulated further investigation and prolonged the study. Much remains to be done before a complete knowledge is attained of the factors that bring about the differential functioning of these two sorts of pollen grains. Definite evidence has been obtained that even with genetically uniform stocks the pollen of some plants behaves differently under adverse treatments from that of others. Clearly the conditions under which pollen is produced are important factors in determining its subsequent reaction. Since the conditions operating throughout the maturing of pollen are largely beyond artificial control it is evident that physiologically identical pollen samples are not to be had from place to place or from season to season.

It is still not possible to outline a treatment that will produce a predictable alteration in the proportion of waxy to horny functioning pollen grains. However, some of the difficulties encountered in these investigations and the means employed to surmount them may be of interest to those undertaking similar studies in the physiology of pollen.

In the course of this study extensive data have been assembled, and it is the purpose of the present paper to summarize this accumulated information.

#### REVIEW OF LITERATURE

The literature on the effect of pollen storage on Mendelian ratios is brief, but several papers on gametic selection have a direct bearing on this subject.

Knowlton (13),<sup>3</sup> in a study of pollen longevity, found that the maximum duration of fertilizing power of pollen of Golden Bantam sweet corn was from 70 to 80 hours. He concluded that the death of corn pollen is caused by desiccation but pointed out that moisture is not the only important factor since life is not greatly prolonged by storage under conditions which retard evaporation. He failed to obtain seeds with pollen stored over calcium chloride at a temperature of 34° C., but his shortest period of storage in this experiment was 6 hours.

In several of Knowlton's experiments the percentage of functioning grains was higher after a long period of storage, as, for example, when pollen was stored at a humidity of 80 to 90 percent at 20° C. With pollen treated in this manner he obtained the following percentages of fertilization: 24 percent at 6 hours, 67 percent at 24 hours, 82 percent at 30 hours, 5 percent at 36 hours, 23 percent at 48 hours, and 6 percent at 54 hours. These percentages were obtained by counting the fertilized and unfertilized ovules in each ear, but only the number of ears is given. In the experiment just mentioned, two ears were used for each period with the exception of the 48-hour period, when three ears were pollinated, and of the 54-hour period, in which only one ear was pollinated. From the published data no estimate of errors is possible, and probably none is justifiable because of the great variability, but it is of interest to observe that apparently certain storage conditions are favorable to longevity of corn pollen.

<sup>3</sup> Reference is made by number (italic) to Literature Cited, p. 120.

Knowlton (13, p. 787) states:

The results from the storage experiments with pollen were exceedingly variable. Many factors that are difficult to control may influence the results. Pollen produced in different seasons and under diverse conditions is, in some way, physiologically different, as is shown by differences in water content, the different optimum sugar concentrations required for germination, and the variation in duration of life.

Holman and Brubaker (11a) tested the longevity of pollen of about fifty species when stored in the dark under various degrees of humidity. Their measure of vitality was the ability of the grains to germinate in sugar solutions. Certain difficulties were encountered in obtaining uniform samples of pollen, which they believe could be obviated by mechanical mixing. However, they noted with several species that the lots of pollen contained some grains much longer lived than others. They state:

In brief, it may be said, then, that in the case of many pollens each lot consists of a relatively large number of grains which retain their viability for a relatively short period, and a smaller number, constituting generally less than 10 percent of the whole, which survive storage several times as long as the others.

They note also that the pollens of the grasses differ from those of most other plants tested in that the death of grass pollen is caused by the loss of water, whereas lowering the water content extends the life of many other plants.

Brink (3) reports unpublished experiments of Thelma McWilliams on pollen storage that not only failed to increase the proportion of functioning waxy pollen but in fact showed the opposite effect. In these experiments aging pollen tended to lower the proportion of effective waxy pollen grains.

Sprague (21) investigated the time required for germination by different sorts of maize pollen on excised silks. He found that on the average waxy pollen grains required 3.8 minutes longer for germination than did the horny pollen grains. This time differential was very much greater when the pollen grains were carrying the sugary gene in addition to the waxy one. It is quite possible that the effects observed from pollen storage are brought about by changing the time required for germination by different sorts of pollen grains.

Ciesielski (6), working with hemp (*Cannabis sativa* L.), conducted experiments on the alteration of the sex ratio resulting from the use of stored pollen. After finding a minor difference in the ratio between seeds resulting from early morning pollinations and those from pollinations made in the afternoon, he tried storing pollen in paper. He states (6, pp. 10-11):

In view of the success of these experiments in fertilization I took up, in 1877, six female plants with their roots, before they had fully produced their flowers, and transplanted them into pots: as soon as the plants were well-rooted I transferred the pots to the windows of two rooms looking south, three pots in each room. Thereafter, with a brush, I fertilized the three plants in one room with pollen which I collected from anthers just dehiscing and not yet fully open (this pollen I shall refer to as "fresh"), and the three pots in the other room I also fertilized by means of a brush, but with pollen taken in the morning and kept in paper till evening. The three plants fertilized with "fresh" pollen produced 120 seeds; the three fertilized with "stale" pollen produced 96 seeds.

In 1878 I sowed in my garden with great care the seeds of the two lots separately, at distances of 20 centimetres. The 120 seeds derived from fertilization with fresh pollen produced 112 plants, of which only six were female, all the rest being male. But the other 96 seeds, sprung from fertilization by stale pollen, produced 89 plants, every one of which was female. I have several times since repeated the experiment, always with a similar result.

The actual experiment reported leaves no room for doubt as to the different sex ratio in the two lots of seed, but since no mention is made as to the common source of the pollen it does not follow that the results are due to the effects of storage. However, in view of the statement of the results of repetitions, there is little reason to question the effects of pollen storage on the sex ratio in hemp.

Mangelsdorf (16, 17) has shown that certain types of maize pollen can be sorted mechanically by means of bolting sieves. Although size differences in pollen grains have no direct bearing on storage, the fact that such differences do exist must be taken into account in sampling.

Bond (1), working with shape and color of seeds in peas, has shown that the Mendelian proportions of these characters are altered by pollen storage, although he made no direct storage experiments. In his investigations pollen was applied to mature and immature stigmas. That on the immature stigmas presumably was stored until the stigmas were receptive.

Christie and Wriedt (5) found a seasonal variation in the Mendelian ratios of certain color factors in pigeons and chickens, but they failed to discover any seasonal effect on the ratios of yellow and green or tall and dwarf peas.

Correns (8a) investigated the effect of aging pollen on the sex ratio in *Mercurialis*. Pollen was stored for various periods up to 120 days, and the seeds resulting from the treated pollen were planted with a control obtained by the use of fresh pollen. In general it was found that the older the pollen the higher the proportion of male to female plants in the progeny.

Malhotra (14, 15) showed that the sex ratio in asparagus could be altered by pollen storage. Certain treatments increased the ratio of male to female plants whereas other treatments reversed this effect.

Sisa (20) found that pollen vitality in cucurbits changed rapidly with age. He observed that the germinating power of pollen in flower buds increased from early morning until 9 a. m. of the day preceding blooming. From this time until the flowers opened the germinating power decreased slowly, but at 10 a. m. of the day of blooming there was a sudden decrease in pollen vitality.

Nakamura (18) records a remarkable fluctuation in the occurrence of imperfect pollen grains in *Impatiens balsamina* L. associated with changes in the air temperature. He found that imperfect pollen grains varied from 9 to 100 percent, the imperfections being due to irregularities in meiosis. Normal cell divisions took place when the maximum air temperature was less than 30° C., but at temperatures above this point there is nonconjunction, nondisjunction, lagging, grouping, etc. Irregularities of this nature are not present to complicate the problem of differential functioning of maize pollen. Classification of pollen samples by staining with iodine shows great regularity in the ratio of waxy to horny grains, and it must be concluded that these two sorts of pollen are formed in the expected proportions.

Haigh and Lochrie (11), in a study of seasonal effect on Mendelian ratios in *Vigna*, found a strong tendency for the plants to produce an excessive proportion of recessive forms in the first flowers and a corresponding excess of the dominant forms in the last flowers.

Sasahi (19) cultivated pollen in various nutrient media and also studied how long it would retain its power to form pollen tubes. He

concluded that maize pollen was preserved best at 50 percent humidity. He found that the duration of the preservation of fertilizing power does not always coincide with the preservation of the ability to form pollen tubes and concluded that the duration of the real fertilizing power must be determined by actual pollinations.

Gotoh (10) shows there are many factors that influence germination in artificial media. Quality of the glass, hydrogen-ion concentration of the medium, and dryness of the pollen are all important factors. Bursting is influenced more by the pH of the medium than by osmotic pressure. He found that different varieties of maize vary greatly in the percentage of pollen grains that will germinate. He found also that pollen with from 10 to 50 percent of shrunken grains germinated better than turgid pollen and that the highest percentage of germination occurred near the point of greatest bursting.

### METHODS

As this investigation progressed, several methods of treating pollen during storage were tried. These included exposure to direct sunlight for various time intervals and to artificial light of several intensities, storage in high and in low temperatures, storage in atmospheres of oxygen and of carbon dioxide and storage for 17 and for 24 hours in the dark.

Rather early it became apparent that the question of uniform sampling might be important. Accordingly an attempt was made to determine whether random sampling could be achieved in composite samples and further to analyze the factors that might affect subsampling. By means of an air blast in vertical long glass tubes, an attempt was made to discover whether the two sorts of pollen grains differed in weight; fine wire bolting sieves were used in an effort to determine differences in size, and specific gravity separations were made with solutions of alcohol and chloroform. In these experiments the sorted pollen grains were classified microscopically and duplicate samples were used in pollinations as a check on differential functioning.

In the earlier experiments all pollinations were made on an individual plant basis. Pollen was collected separately from heterozygous plants, and the identity of each sample was retained for all treatments. This procedure involved a control pollination for each plant furnishing pollen, a practice that doubled the work not only in making and recording pollinations but also in the subsequent classification of seeds. There was a further disadvantage of this system in that many of the pollen treatments proved lethal and their control ears from fresh pollen were consequently useless.

To obviate these disadvantages bulk samples of pollen were collected from as many as 50 heterozygous plants. The samples were sieved to remove anthers, then thoroughly mixed by pouring back and forth, and subsequently divided into as many subsamples as the quantity permitted. Care had to be exercised in mixing, as the grains tended to clump and even to become pasty if too large quantities were collected and the mass became too dense.

This method of handling pollen simplified field practice, but with the exception of a single small group all the ears resulting from the use of bulk pollen samples showed significant reductions in the pro-



portion of waxy to nonwaxy seeds irrespective of pollen treatment. It seemed reasonable to ascribe this unexpected result to some condition arising in the method of sampling, and until this possibility could be investigated the method of bulk sampling was abandoned. Subsequent attempts to obtain low percentages of waxy seed from bulk pollen samples have been unsuccessful, and the earlier results remain without satisfactory explanation.

Not only did the screening of numerous individual samples consume much time, but in many cases the accumulation of large numbers of damp anthers reduced materially the quantity of usable pollen, besides introducing a possible source of variability by affecting the moisture content of the pollen grains.

In the work of the last few years the tassels of pollen parents were enclosed in two bags, one within the other. The inner bag, slightly smaller than the outer one, was perforated with numerous fine holes too small to permit the passage of anthers. By this means the pollen sifted into the outer bag almost as soon as shed and no sieving was required. This pollen was taken from the field in tassel bags, transferred to small pill boxes, and given treatments while in these boxes. For pollinating the covered boxes were taken to the field, and in most cases two ears were pollinated from each box.

All female parents had their tassels removed and the silks were cut back 24 hours before pollination to provide uniform brushes of silks. Many days before silking, the ears were covered with open-ended bags closed at the outer end with flexible metal strips and closely fastened to the plant with copper wire. The technique employed on the female plants has not varied over the period of this investigation, and from the hundreds of seedless cobs obtained from many of the pollen treatments it was apparent that the error due to contamination was gratifyingly low.

Several stocks of waxy were used, but all of these have been in cultures of the Bureau of Plant Industry for many years and their behavior under ordinary conditions is well known. In the last 3 years this investigation has been limited to one stock, chiefly because that stock possessed large seeds capable of rapid classification. In the earlier work multiple-eared stocks with numerous small seeds were used, but since analysis showed no influence of the female parent and since the chances of errors in bookkeeping, to say nothing of the failures of lower ears to produce seed, were greater in these strains, they were discarded.

The experimental work was carried on at the Arlington Experiment Farm, Rosslyn, Va., and at Lanham, Md. This fact is mentioned, as the results from the two places were not always alike even in the same year. No question of seed stocks nor of personal equation is involved, however, as the seed and the personnel were the same in both localities. The geographical separation of the experimental work adds one more complication to the presentation of data already complex because of numerous time intervals, sampling techniques, and types of control.

#### ANALYSIS OF SAMPLING

The results of the effect of storage on Mendelian ratios in functioning pollen depend for their accuracy on the possibility of subdividing large samples of pollen into smaller subsamples.

Where pollen is collected in bulk from 25 or more heterozygous plants and is subsequently subdivided into subsamples for various treatments it is essential that the subsamples be alike at the start of the treatments.

Similarly, where pollen is collected separately from individual plants and this pollen is subdivided into lots, one to be applied as a fresh control and the others to be subjected to different treatments, the several lots must be random samples of the original collection.

After treatment there is no method for determining whether or not the control and treated subsamples were alike at the time of subdivision. It can be determined that an array of ears obtained from a given lot of pollen used fresh is a random one, and it may be assumed that the corresponding lots in the treated series were random also; but this does not provide a measure of a possible selection. It is quite possible in pouring subsamples to obtain two sets, both distributed at random about their respective means; yet the two means may differ unless care has been exercised to draw the samples for each set at random.

To provide a measure of the randomness of subsampling, each sample of pollen has been used to pollinate two ears. As is to be expected, especially where adverse treatments were given the pollen, there were many cases where only one ear of the pair produced seed. However, enough cases of paired pollinations were obtained to permit a measure of the randomness of subsampling. To these may be added certain ears where the butts and tips were classified separately.

In one experiment a bulk sample of pollen was collected from about 25 plants, thoroughly mixed, and subdivided into 10 lots of approximately equal size, which were subjected to a secular selection by the following method: The 10 subsamples were poured from a bag containing the bulk sample; the first subsample was numbered 1, and the last lot of pollen to leave the bag was numbered 10. These lots were used to pollinate 20 ears, each lot being used on 2 ears. The subsamples were applied consecutively, lot 1 being applied to ears *a* and *b*, lot 2 to ears *c* and *d*, etc. By this means it was hoped to obtain some knowledge as to whether or not the method of pouring subsamples involved an unconscious sorting of the pollen grains.

Only 17 of the 20 pollinations were effective. The correlation between the number of a subsample and the percentage of waxy seeds obtained is  $\rho = -0.123 \pm 0.169$ , clearly indicating that the method of sampling did not introduce any regular selection of the pollen grains.

When the variance of the population of paired ears resulting from the use of fresh pollen is subdivided into its several elements it is found that there is no difference between subsamples in the proportion of waxy to horny pollen grains that function in the production of seed. There is a correlation of 0.5 between the pollen grains within subsamples, but the number of samples is too small to attach any significance to the coefficient. The apportioned variance for this population is given in column 2 of table 1.

As a comparison with the above population, the variance is apportioned for two similar populations of paired ears resulting from the use of fresh pollen, where the pollen, instead of being collected in bulk from many plants, was collected separately from each plant and each collection was used on a single pair of ears. The mean squares for these two populations are shown in columns 9 and 10 of table 1.

It will be observed that the mean square of the means of subgroups is but two-thirds of that found when the bulk sample of pollen was used.

It was not to be expected that the mean square of the means of subgroups from pollinations with individual plant samples would be lower than the mean square of the means of subgroups from pollinations with subsamples of composite collections. Composite samples never should have mean squares larger than those of the individual samples, and the expectation would be for lower mean squares. Since the difference between the mean square for the composite sample (12,043.74) and that for the combined populations in columns 9 and 10 of table 1 (8,012.68) is not significant, the result may be attributed to chance.

Although in both populations from individual plant samples the mean square of the means of subgroups exceeds that of the pair members from their mean, the differences are not significant, and it may be concluded that for these populations there is no reason to believe that the ratios of the two kinds of pollen grains were more nearly the same within subsamples than between subsamples.

Three other populations resulting from the use of fresh pollen are available for analysis. In these three populations the subsamples consist of the butts and tips of the ears. Two of the populations are from the use of composite collections of pollen, the third being from individual plant pollinations. No manual division into subsamples is involved in these three populations, and in the population from individual plant pollinations the mean square of the means of subgroups measures the differences between male parents.

The mean squares are given in columns 6, 7, and 11 of table 1. In all three populations the mean square of the means of subgroups exceeds that of the members of the pair from their mean, and in two of the populations the differences are clearly significant. In the third (column 6) the difference just borders on significance. Were the comparison limited to the population given in column 11 it would be a fair inference that male parents differed one from the other, but with the composite samples no such interpretation is possible.

For purposes of comparison a single population has been included (column 13) where the variability is limited to the ovules, the pollen having been all of one kind—waxy. In this population the mean square of the means of subgroups (composed of butts and tips) is actually less, though not significantly so, than that of the butts and tips from their mean. This is the only case of the 12 presented in table 1 where the means of subgroups are less variable than their components. It is clear that the difficulties in sampling and the observed correlations between butts and tips and between paired ears from the same pollen subsample are confined to the pollen. Since the composite samples are less satisfactory in this respect than the individual plant samples, it is evident that the results flow from physiological rather than genetic differences in pollen. This conclusion receives further support when the populations of ears resulting from the use of stored pollen are examined.

There is one population resulting from the use of stored pollen collected from individual plants. The mean squares for this population are given in column 12 of table 1 and may be compared directly with those in column 11, which are for a population from the same

TABLE 1.—Apportionment and analysis of variance of percentage of waxy, showing the mean squares for the several groupings

Variation	Composite pollen sample split into subgroups										Individual pollen samples collected from separate male parents		
	Pollen, after indicated treatment, applied to paired ears					Ears resulting from pollen used after indicated treatment, classified separately for butts and tips					Fresh pollen applied to paired ears		
	Fresh	Stored at 37° C. <sup>1</sup>	Stored at 10° C. <sup>1</sup>	Stored in oxygen <sup>1</sup>	Fresh Lundum 1926	Fresh Arlington 1926	Stored in sun 5 hours				Fresh	Stored in sun 7½ hours	Male waxy, female heterozygous
1	2	3	4	5	6	7	8	9	10	11	12	13	
Apportionment:													
Total seeds.....	2,488.31	2,160.98	2,143.24	2,340.42	2,500.20	2,465.05	2,482.46	2,477.11	2,473.27	2,458.82	2,497.39	2,500.04	
Seeds on ear.....	2,474.48	2,154.51	2,133.13	2,339.02	2,486.96	2,455.82	2,464.77	2,465.98	2,464.03	2,442.98	2,433.87	2,498.24	
Ears.....	8,021.81	5,444.39	6,757.41	3,038.91	6,031.29	4,824.11	7,430.89	7,328.34	7,865.02	7,111.36	19,669.99	2,929.06	
Pair members from their mean.....	4,574.43	3,841.87	3,227.89	2,606.18	3,205.91	77.20	1,734.30	7,201.34	7,465.04	2,789.93	6,126.19	3,027.46	
Means of subgroups.....	12,043.74	7,848.15	12,492.90	3,687.99	9,092.13	10,363.17	13,443.96	7,408.03	8,285.00	11,576.83	33,460.03	2,879.78	
Analysis:													
Means of subgroups.....	.483	.356	.678	.174	.520	2.43	.102	.018	.052	.709	.845	.024	
Pair members from their mean.....	>.05	>.05	>.01	>.05	>.01	>.01	<.01	>.05	>.05	<.01	<.01	>.05	
Significance of z.....	14	11	22	16	26	14	38	22	42	62	112	236	
Primary groups.....	5,216	7,780	9,609	7,501	6,094	3,336	10,335	9,179	23,952	16,912	30,120	69,550	
Total seeds.....	46.50	31.58	31.11	37.36	50.42	44.03	45.78	45.10	44.80	43.65	48.36	49.96	
Waxy seeds.....													

<sup>1</sup> Stored for 2½ hours and applied 3½ hours after collection.

pollen parents where the pollen was applied immediately following collection. The enormous increase in the mean square between ears will be noted in this population of ears from stored pollen. This may mean either that the stored samples of pollen have not been accorded uniform exposure to sunlight or that the samples reacted differently to exposure. Probably the high intraclass correlation ( $\rho = 0.701 \pm 0.046$ ) is the result of both factors. It should be noted, however, that the mean square of the pair members from their mean is also excessive in this group of ears. Thus the tips and butts of the same ears tend to differ from each other, indicating a differential functioning of the two sorts of pollen tubes in long and short styles.

A similar situation is encountered in a population of ears resulting from the use of pollen stored in the sun where a composite collection of pollen was subsampled. The mean squares for this population are given in column 8 of table 1.

In columns 3, 4, and 5 of table 1, the mean squares are given for a single composite sample of pollen, which was subdivided into small samples that were used in each case to pollinate a pair of ears. All three lots of subsamples were drawn at random from a single bulk collection, treated for  $2\frac{1}{2}$  hours, and applied  $3\frac{1}{2}$  hours after collection. In all three cases the waxy percentages are low although the lots stored at  $37^\circ$  and  $10^\circ$  C., respectively, do not differ significantly, the  $\chi^2$  of the difference of these two populations being 3.31 ( $P = 0.069$ ). The samples stored in oxygen have a significantly higher percentage of waxy seeds than either of the two temperature series. The oxygen treatment differs from the  $37^\circ$  treatment, with a  $\chi^2$  of 52 ( $P < 10^{-8}$ ), and from the  $10^\circ$  group, with a  $\chi^2$  of 97 ( $P < 10^{-8}$ ). The oxygen series shows rather conclusively that the subsampling was random, whereas the  $10^\circ$  treatment shows evidence of an intraclass correlation.

If the  $37^\circ$  and the  $10^\circ$  C. populations are combined, on the assumption that the storage treatments resulted in no differential behavior, and the variance of this combined population is apportioned, it is found that the mean square of the means of pairs is 10,477.58 and that of the pair members from their means is 3,420.78. The ratio of the two is 3.062, with  $P$  lying between 0.01 and 0.05. There is, therefore, just a possibility that the pairs of pollinations in this temperature series are not random subsamples of the original composite sample.

Further analysis of variance is possible in the case of a single population where ears were pollinated in pairs and the butts and tips of each ear were classified separately. The pollen in this case was a composite sample split into small subsamples and these were stored in sunlight for 5 hours. Each subsample was applied to two ears. The mean squares for duplicate samples used fresh are given in column 7 of table 1. Not all the paired pollinations were successful, and the mean squares for all the ears irrespective of pairs are given in column 8.

Limiting the population to the ears where paired pollinations were successful and apportioning the variance into its component elements, it becomes possible to compare the variance of subgroups based on butt and tip sampling with the variance of subgroups composed of paired ears.

The data are as follows:

Apportionment of variation:		Mean square
Total seeds.....	-----	2, 467. 89
Seeds on butts and tips.....	-----	2, 458. 60
Seeds on ear.....	-----	2, 457. 08
Butts and tips.....	-----	5, 172. 66
Ears.....	-----	8, 802. 46
Butts and tips from their mean.....	-----	1, 845. 34
Means of subgroups of butts and tips.....	-----	8, 802. 46
Ears from their pair mean.....	-----	5, 900. 78
Means of subgroups of paired ears.....	-----	12, 284. 48
Analysis of variation:		Number
Primary groups of butts and tips.....	-----	24
Subgroups of butts and tips.....	-----	12
Primary groups of whole ears.....	-----	12
Subgroups of paired ears.....	-----	6
Total seeds.....	-----	6, 456
		Mean percent
Waxy seeds.....	-----	44. 3

Comparing the mean squares of this restricted population with those obtained for all the ears as given in column 8 of table 1, it is seen that the agreement is fairly close in all categories other than the mean squares of the means of subgroups composed of butts and tips. In the unrestricted population this is 13,443.96, and in the limited population it is only 8,802.46. The difference is not significant, but apparently in limiting the population to paired ears some of the variability was eliminated. In the restricted population it is seen that the intraclass correlation between butts and tips is significant,  $z$  equaling 0.78 and  $P$  being less than 0.01. No such correlation can be demonstrated for the paired ears, even though the mean square of the means of subgroups exceeds that found for the means of subgroups composed of butts and tips. The mean square of the individual ears from their pair mean is also larger than the mean square of butts and tips from their mean, and  $z$  equals 0.367,  $P$  being greater than 0.05. The difference between the two intraclass correlations, as measured by  $z$ , is  $0.415 \pm 0.398$ .

From the analysis of the variance of the percentage of waxy seeds in butts and tips of these ears, it is clear that the variation between butts and tips is much lower than that between individual ears despite the fact that the pollen was a composite sample. No such condition can be demonstrated between paired pollinations, although there is evidently a tendency in this same direction since the mean square of the means of pairs is twice that of the members of the pair from their mean. Just why the butt and tip of an ear should be more nearly alike than a pair of ears pollinated with the same pollen is not clear. It might be urged that this evidence indicates an influence of the female parent, which, of course, in these cases, was homozygous for waxy and could not contribute directly to variation in waxy percentage unless the hypothesis of selective fertilization is invoked. Other evidence of the influence of the female parent is discussed later.

Not only is the variance of the means of paired ears relatively high, but the variance of the butts and tips from the mean of the ear is too low, clearly indicating an ear individuality.

From a consideration of these mean squares for the several sorts of groupings it is apparent that there is a real problem of subsampling

involved in these experiments, and it is indicated further that the most effective method of subsampling is to collect pollen separately from each male parent. In following this practice each sample must be used once to provide a fresh control, a condition that adds to the field work and subsequent classification. However, experience with composite samples representing the output of many plants has proved so unsatisfactory and of such doubtful reliability that for the last few years all pollinations in these investigations have been made on an individual plant basis.

This method does not wholly eliminate sampling difficulties, as the individual plant collection must be subdivided for various treatments; however, it is the best method possible under the conditions. A certain portion of the discordant results encountered throughout these investigations and the large errors so frequently involved in the populations of ears may properly be ascribed to difficulties in sampling masses of pollen.

#### COMPARISONS BETWEEN WAXY AND HORNY POLLEN GRAINS

##### SIZE

Two methods have been used to determine whether waxy pollen grains differ in size from horny pollen grains. In one method the stained grains were measured under the microscope by means of an eyepiece micrometer, but since there is the possibility that the two forms of starch may expand unequally in the staining medium, thus giving spurious results, an effort was made to separate the two sorts of grains by means of sieves.

A nest of sieves was made up of fine wire bolting screens having, respectively, 140, 170, 200, 220, and 270 wires to the inch. Although the best available screen cloths were procured and the most uniform areas of the material were selected for the sieves the apertures were quite variable. The mean sizes of the apertures of the sieves as given by the manufacturer were 0.105, 0.088, 0.074, 0.062, and 0.053 mm, respectively, but in experiments of this nature the aperture of maximum size in each sieve is important. The sieves are referred to throughout the text by the number of wires to the inch, which is the manufacturer's designation.

Some difficulty was found in the mechanics of sieving, since many pollen samples tended to clump if agitated for a few minutes. A few samples were obtained where the pollen sifted through the sieves almost like dry sand. As pollen grains differ greatly in size from plant to plant, possibly because of differences in moisture content, the samples used in the sieve tests were confined in each case to single male parents.

After separation, the pollen grains left upon each sieve were used to pollinate plants homozygous for the waxy gene, and in some cases samples were classified microscopically. These two methods of determining possible differences in size do not necessarily lead to the same result, for it is quite possible that differences in ability to effect fertilization might exist in the different size groups.

Table 2 shows the most complete series of ears, representing six sieve sizes, obtained from a single sample of heterozygous pollen. There is some indication that the intermediate pollen sizes (as determined by the size of the sieves) have the highest proportion of

functioning waxy pollen grains. However, the duplicate pollinations from the same sieve sizes gave rather widely different results, and the errors are such that no definite conclusion can be reached as to the certainty of this observation. It does seem clear that there is a significant deficiency of functioning waxy pollen grains in the smallest size group. Whether this deficiency indicates that the waxy grains were retained on the sieves of larger aperture or that the small waxy grains did not effect fertilization so well as the small horny grains cannot be determined from these data.

TABLE 2.—Waxy and horny seeds obtained when pollen (single sample from plant 2632) lodging on the various sieves was used on ears homozygous for the waxy gene

Female plant no <sup>1</sup>	Pollen from screen no.	Seeds obtained			
		Total	Horny	Waxy	
		Number	Number	Number	Percent
305.....	140	428	217	211	49.3±1.6
306.....	140	114	61	53	46.5±3.2
303.....	170	427	186	241	56.4±1.6
304.....	170	334	164	170	50.9±1.8
297.....	200	622	265	357	57.4±1.3
295.....	220	162	83	79	48.8±2.7
294.....	270	440	222	218	49.5±1.6
293.....	(?)	59	37	22	37.3±3.5
Total.....		2,586	1,235	1,351	52.24±.66

<sup>1</sup> 305-306 and 303-304 duplicate pollinations;  $\chi^2$  between the combined 305 and 306 and the combined 303 and 304=3.56 ( $P=0.057$ ),  $\chi^2$  between plant 293 and the combined 303 and 304=6.135 ( $P=0.013$ ).

<sup>2</sup> Pollen that passed through all sieves.

TABLE 3.—Analysis of pollen ratios and sizes in samples of sieved pollen from individual plants

Source of pollen and treatment	Seeds from pollinations		Difference between seeds and pollen		Pollen classified microscopically								Red pollen—blue pollen	
							Blue-staining				Red-staining		Differ- ence in size	S. E.
	Waxy <sup>1</sup>	S. E.	Waxy	S. E.	Waxy	S. E.								
							Size	S. E.	Size	S. E.				
Plant 7133. Unsieved..... Caught on— Sieve 140..... Sieve 200..... Sieve 270..... Bottom.....	Pct. 50.3 Pct. 53.1 53.4 52.3 52.6	Pct. 3.1 2.2 2.2 2.2 2.2	Pct. ----- ----- ----- ----- -----	Pct. ----- ----- ----- ----- -----	Pct. ----- ----- ----- ----- -----	Pct. ----- ----- ----- ----- -----	Mm 0.08512	Mm 0.00060	Mm 0.08496	Mm 0.00049	Mm -0.00016	Mm 0.00078		
Plant 7505 <sup>2</sup> Unsieved..... Caught on— Sieve 140..... Sieve 170..... Sieve 200.....	Pct. 51.4 44.4 44.0 43.8	Pct. 2.1 11.7 1.3 2.5	Pct. 0.0 5.3 4.3 5.8	Pct. 3.04 11.90 2.55 3.32	Pct. 51.4 49.7 48.3 49.6	Pct. 2.2 2.2 2.2 2.2	Mm .09840	Mm .00072	Mm .09392	Mm .00064	Mm -.00448	Mm .00096		
Plant 3425: Caught on— Sieve 170..... Sieve 200.....	Pct. 56.0 41.5	Pct. 1.3 1.3	Pct. ----- ----- -----	Pct. ----- ----- -----	Pct. ----- ----- -----	Pct. ----- ----- -----	Mm .10144	Mm .00161	Mm .10176	Mm .00096	Mm .00032	Mm .00194		
Plant 7256: Caught on— Sieve 140..... Sieve 170.....	Pct. ----- -----	Pct. ----- -----	Pct. ----- -----	Pct. ----- -----	Pct. ----- -----	Pct. ----- -----	Mm .07872	Mm .00060	Mm .07992	Mm .00092	Mm .00120	Mm .00110		
	Pct. -----	Pct. -----	Pct. -----	Pct. -----	Pct. -----	Pct. -----	Mm .07856	Mm .00066	Mm .07772	Mm .00056	Mm -.00084	Mm .00086		

<sup>1</sup> Percentage of waxy seeds obtained when the pollen lodging on the sieve indicated was used on ears homozygous for the waxy gene.



No other pollen sample provided so many sizes; the majority of the samples lodged on only two or three sieves.

Waxy-horny ratios, together with micrometer measurements of the pollen grains, were determined for three samples of sieved pollen that was classified microscopically. The most complete series is that for the pollen sample from plant 7133, the data for which are given in table 3.

In this sample there is no evidence that the waxy pollen grains differ in size from the horny grains or that the sieves were at all effective in sorting the two kinds of grains.

As a check on the effect of the staining fluid on the size of grains, 50 grains were measured dry from the sample left on sieve 200. These grains, of course, could not be classified into waxy and horny, so the comparison must be made with the mean size of the stained grains from the same sieve. The mean size of the dry grains was found to be  $0.08912 \pm 0.00056$  mm, whereas the mean size of the stained grains from this same sieve was  $0.08364 \pm 0.00044$  mm. The difference ( $0.00548 \pm 0.00071$ ) is clearly significant but is in the direction of a shrinkage of the stained grains.

In the second of these samples the pollen from each sieve was subdivided, one lot being classified and measured under the microscope, the other being used to pollinate homozygous waxy ears. The data are shown in table 3.

The classifications made under the microscope show that all samples closely approximate 50 percent waxy, the widest departure being found on sieve 170 where the percentage (48.3) departs from 50 with  $\chi^2$  only 0.5 ( $P=0.479$ ). In the unsieved pollen the blue-staining grains were 4.8 percent larger than the red-staining grains—a difference that is 4.75 times its standard error, giving  $P=0.000002$ . However, there is some evidence that the unsieved pollen was not a random sample of the sieved pollen, for the mean size of the unsieved grains was  $0.09616 \pm 0.00053$  mm, whereas the mean size of the pollen grains stopped by the sieve with the largest apertures (140) was  $0.09272 \pm 0.00035$  mm. The difference ( $0.00344 \pm 0.00063$ ) is more than 5 times its standard error. No explanation can be offered for this discrepancy in size.

When the ears resulting from the use of these several samples of pollen are examined an entirely different situation is encountered. The percentage of waxy seeds obtained from unsieved pollen is exactly the same as the percentage of waxy pollen found in counting the pollen grains under the microscope. The exactness of this agreement is, of course, merely a coincidence. Only 18 seeds were obtained from the pollen used from sieve 140, and the percentage is included in the table simply for completeness. The percentages of waxy in the other two samples are both significantly lower than that found in the unsieved pollen, indicating that, even though the actual proportion of waxy to horny pollen grains remains the same in all size groups, in the small sizes waxy grains are less effective than horny grains in accomplishing fertilization.

There are no significant differences between the percentages of waxy as determined by using the pollen to produce seeds and as determined by microscopic examination.

The third sieved sample (table 3) provided only two sizes, the pollen all lodging on sieves 170 and 200. The difference in the per-

centage of waxy pollen grains ( $14.5 \pm 1.84$ ) is clearly significant, the pollen passing to sieve 200 having the lower percentage of waxy grains. However, within the sieved samples there was no significant difference in size between the two sorts of grains.

One other sample of pollen was sieved and the sizes of grains were measured (table 3), but through inadvertence no record was made of the proportion of waxy to horny pollen grains.

This sample serves to emphasize the fact that within a sieve class the two sorts of pollen grains are of the same size. With this sample, to provide a check on possible distortion of size by the staining medium, dry pollen was measured. That from sieve 140 had a mean size of  $0.07364 \pm 0.0078$  mm and that from sieve 170 had a mean size of  $0.07612 \pm 0.0006$  mm. The mean size of the dry grains on sieve 170 exceeded that of the dry grains on sieve 140 by an amount that was 2.3 times its standard error. This relationship is reversed in the stained grains, the mean size of the red grains from sieve 140 exceeding that from sieve 170 by an amount that was 3.0 times its standard error. Such results indicate that despite the manufacturer's rating there is no effective difference in the sizes of the apertures in sieves 140 and 170.

In another sample, not shown in table 3, the pollen all lodged on sieves 170 and 200. That on the sieve of larger aperture proved to have by count under the microscope  $53.5 \pm 1.57$  percent waxy grains and that on the sieve of smaller aperture  $46.0 \pm 0.95$  percent waxy grains. The difference ( $7.5 \pm 1.83$ ) is over four times as large as its standard error.

In summarizing the evidence for the pollen classified under the microscope, it would appear that some plants produce pollen of which the horny grains are somewhat more likely to pass a sieve of a given size than are the waxy grains. It has been demonstrated that there is not always a corresponding difference between the sizes of the two sorts of grains. Measurements of pollen grains in unsieved samples indicate that it is questionable whether the sieves functioned as selectors of size. The observed results could be explained by slight differences in stickiness between waxy and horny grains.

From the samples used in pollinations there is strong reason to conclude that in pollen grains of the smaller sizes the waxy pollen is at a disadvantage in effecting fertilization.

Before leaving this subject it should be noted that the pollen grains from the several samples differ significantly in size. They range from 0.07 to 0.10 mm, with errors in the third decimal place.

#### WEIGHT

For the purpose of disclosing differences in weight between waxy and horny pollen grains, samples of pollen from heterozygous plants were separated by means of an air blast.

The apparatus consisted of a vertical glass tube about one-half inch in diameter and 30 inches long, which was bent at the upper end to deflect the floating grains into a suitable container. The lower end was closed with a fine-meshed bolting cloth and inserted into the neck of a large flask. Air under pressure was admitted to the flask by means of a side connection. The flask served to smooth convection currents and provided a more even air pressure on the bolting cloth.

The pollen to be tested was placed on the bolting cloth and with the increase in air pressure rose through the tube and spilled into the container. No attempt was made to record air pressure, which was controlled by a needle valve and increased as required to drive over the last grains.

The pollen grains that floated through the tube with the lowest pressure were kept separate from those that rose through the tube only with the highest pressure. Samples of the first and last lots were stained with iodine and counted. The bulk of the pollen was applied to silks of plants homozygous for waxy for an eventual seed count.

The two sorts of grains in both the light and heavy samples were essentially equal in number and the  $\chi^2$  of the difference between the light and the heavy weight was 0.80 ( $P=0.37$ ).

Only four samples of pollen produced ears from both the heavy and the light weight. In the most successful of these, three ears were produced from each of the weight groups. Combining the three ears from each group, the  $\chi^2$  of the difference between the ratios of horny to waxy seeds is found to be 3.89 ( $P=0.048$ ) indicating that the air blast did not separate the two sorts of pollen grains.

As a further analysis of these data, 10 ears resulting from heavy pollen were combined, and the mean percentage of waxy seeds was compared with that obtained when 10 ears resulting from light pollen were combined. The heavy pollen gave  $48.11 \pm 0.85$  percent waxy and the light pollen gave  $47.92 \pm 0.57$  percent waxy. The difference between the two groups ( $0.19 \pm 1.02$ ) is not significant. From these data it may be concluded that any difference in the percentage of waxy seeds due to the use of pollen of different weights could not have been greater than 3 percent.

#### SPECIFIC GRAVITY

As a means of determining whether waxy pollen grains differed from horny pollen grains in specific gravity, pollen from a heterozygous plant was placed in a tube filled with a solution of chloroform and alcohol in the proportion of 38 : 6. This solution could be so accurately balanced that the temperature would determine whether the grains would sink or float; a drop of  $1^\circ$  or  $2^\circ$  C. would sink all pollen grains, whereas an increase of a few degrees would bring the grains to the surface. By adjusting the proportions of chloroform and alcohol and holding the tubes under constant temperature, a fairly good separation of pollen grains could be obtained. Pollen grains from the top and bottom of the tube were pipetted off, stained with iodine, and classified under the microscope. Several individual plant samples were tried. In one the floating grains gave 69 horny to 68 waxy and the sunken grains gave 161 horny to 148 waxy. The  $\chi^2$  of the difference between the sunken and the floating grains is 0.114 ( $P=0.74$ ), indicating a chance difference between the two sets. With another sample the floating grains were classified as 1,135 waxy to 929 horny, and the sunken grains gave 536 waxy to 494 horny.

Fifty-five percent of the floating grains and 52 percent of the sunken grains were waxy. The difference of 3 percent has a probability of 0.066. From these tests it may be concluded that the two sorts of pollen grains do not differ in specific gravity.

## ELECTRICAL TEST

An attempt was made to separate the two sorts of pollen grains electrically, but this proved unsuccessful. Two strips of zinc were suspended about one-quarter of an inch apart in a tube containing chloroform and alcohol in which heterozygous pollen was suspended. These zinc poles were connected with a 6-volt storage battery, but no lateral movement of the suspended pollen grains could be detected.

## EFFECT OF VARIOUS FACTORS ON RATIO OF WAXY TO HORNY SEEDS

## TIME OF ANTHESIS

It has been suggested that the effects observed in stored pollen might be explained by a differential maturity of the two types of grains; that a simple differential death rate would account for percentages of waxy seed greater than 50 in stored pollen but would not explain percentages less than 50 when fresh pollen is used.

If waxy pollen grains are in fact immature at anthesis, then the very first pollen shed in the morning should result in ears with the lowest percentage of waxy seeds, and this percentage should increase with time. To test this point an effort was made to collect pollen early in the morning, but on most August days in the vicinity of Washington heavy dews delay pollen shedding until 8 a. m. Even when a few favorably situated plants shed some pollen before this hour, the moisture that accumulated on the spikelets interfered with collection. However, one series beginning at 6:45 a. m. was successful. From this series there is no evidence that the waxy pollen grains are less mature when shed early in the morning than when shed later. The data are shown in table 4.

TABLE 4.—Effect of hour of collection of pollen on percentage of waxy seeds

Hour of collection	Ears	Total seeds	Waxy seeds
<i>A. m.</i>	<i>Number</i>	<i>Number</i>	<i>Percent</i>
6.45.....	6	1,317	46.6±1.1
7:15.....	5	1,554	50.3±.5
7.55.....	7	2,290	46.7±.9
8.30.....	15	6,298	45.5±.8
Mean.....	33	11,459	46.5±.5

TABLE 5.—Effect of hour of collection of pollen from each of 4 plants on percentage of waxy seeds resulting from fresh applications, July 30, 1932

Source of pollen (plant no.)	Waxy seeds from pollen collected at indicated hour		
	8 a. m.	11:30 a. m.	Difference (11.30-8 a. m.)
	<i>Percent</i>	<i>Percent</i>	<i>Percent</i>
1.....	47.3±0.95	51.1±1.12	3.8±1.5
2.....	45.7±1.03	44.7±1.13	-1.0±1.5
3.....	47.9±.97	42.9±1.02	-5.0±1.4
4.....	43.8±.90	45.8±1.09	2.0±1.4

With the exception of the collection made at 7:15 a. m., the ratios of waxy to horny seeds are significantly less than equality but do not differ from one another. In one other case pollen was collected from four plants at 8 a. m. and was applied immediately. A second collection of pollen was made from these same plants 3½ hours later. The results are shown in table 5. Of the four differences, one is three and one-half times its error; but since it is one difference in a series of four, the odds against its being due to chance are about 16 to 1.

The time of day at which the pollen is shed, therefore, seems to bear no relation to the deficiency of waxy seeds resulting from the use of fresh pollen. This deficiency may be due to immaturity, but if so the waxy grains are equally immature irrespective of the time of anthesis. The results accord better with the observations of Sprague (21), i. e., that waxy pollen grains germinate more slowly than horny grains.

#### CONDITIONS OF POLLEN STORAGE

##### STORAGE IN SUNLIGHT

Although in 1925, 1926, 1927, and 1929 pollen exposed for as long as 7 hours to sunlight was capable of functioning in fertilization, similar exposures in 1928 were uniformly lethal, and much shorter periods tried in 1930 and 1931 (some as short as 10 minutes) were practically lethal, only a few seeds being obtained. In 1932 a few exposures for as long as 5 hours were successful. Exposures for shorter periods (15 to 45 minutes) showed no lethal effects. It became possible in 1932 to measure the degree of illumination by means of photronic cells, and it was found that the brightest cloudless summer days in the vicinity of Washington, D. C., registered 12,500 foot-candles in direct sunlight and that the maximum observed in Arizona was 13,000 foot-candles. Under a single layer of glassine paper the radiation was reduced about one-third.

On most days in late July and early August, when these experiments were conducted, the sky is far from cloudless and the illumination varies rapidly from 2,000 to 12,000 foot-candles. Since the experiments were made over a period of from 10 days to 2 weeks in any one year, it is not believed that the lethal effects obtained in the years 1928, 1930, and 1931 were caused by excessive radiation.

While no measurements of radiation were made previous to 1932, records were kept as to whether the day was clear, partly cloudy, or cloudy. So far as these records serve, they fail to support the idea that the death of pollen in 1928, 1930, and 1931 was due to unusually intense radiation. It is more reasonable to assume that in these years pollen was more sensitive to radiation than in the other years. This hypothesis receives support from the fact that pollen from different plants does not react alike to storage treatments.

With a knowledge of these limitations in mind, the results obtained from exposures of pollen to sunlight may be examined.

In 1927 no pollinations were made with fresh pollen, and the comparison for this year is limited to sun storage as contrasted with storage in the dark for the same period of time. The comparisons are all on an individual plant basis, each plant furnishing pollen for a sun and for a dark exposure. All the samples were in glassine bags, and the sun exposures were made on a flat table in direct sunlight. The time intervals ranged from 7½ to 7¾ hours.

Only 13 pairs of pollinations from light-dark treatments were successful. In 12 of the 13 cases the pollen exposed to direct sunlight produced ears with a higher percentage of waxy seeds than the duplicate samples stored during the same period in the dark. The mean excess for the series was  $20.13 \pm 2.01$  percent, despite the fact that the dark series averaged  $50.4 \pm 0.7$ . The mean difference and its error is weighted by  $1/E_d^2$ ; the details of this statistical procedure have been published elsewhere (8).

As part of the series of heat treatments given at Arlington, Va., in 1928, a series of duplicates was exposed to sunlight. Most of these were for periods of  $5\frac{1}{2}$  hours and all proved to be lethal. The only pollen that produced seeds after exposure to sunlight was a composite sample spread in a thin layer in a large uncovered Petri dish resting on a block of ice. Seven ears were obtained from this treatment, which lasted for  $4\frac{1}{2}$  hours. The mean percentage of waxy seeds was  $47.30 \pm 0.73$ . No other treatments given on this day produced ears, so there is no other population with which to compare this percentage. Since, however, the average percentage of waxy seeds from fresh pollinations made at Arlington in this year with composite samples of pollen was  $46.24 \pm 0.78$ , there is every reason to conclude that the sample stored on ice in direct sunlight for  $4\frac{1}{2}$  hours was not affected by the exposure.

In 1929, at Lanham, Md., the length of the period of exposure to sunlight ranged from 6 hours and 40 minutes to 8 hours and 45 minutes, but the individual variability was so great that no conclusions can be drawn as to the relationship of time to the degree of alteration in the waxy-horny ratio.

All pollinations were made on the individual plant basis, and the comparisons are between pollen that was used fresh and pollen that was stored.

The pollinations were made on 2 days, August 5 and 8. August 5 was a bright clear cold day, with radiation probably approximating 12,000 foot-candles, whereas August 8 was cloudy. Only three pairs of pollinations were successful on August 5, all showing a pronounced effect of the exposure to sunlight. The mean difference in the percentage of waxy seeds from stored pollen and that from fresh pollen was  $16.4 \pm 3.3$ , the stored pollen producing the higher percentage.

The pollinations made on August 8 were more successful, doubtless because of the less rigorous conditions prevailing during exposure. The effect of the treatment was far less than that of 3 days earlier, though the difference in the percentage of waxy seeds obtained from stored pollen and that obtained from fresh pollen was  $4.9 \pm 1.3$  percent, in favor of the stored pollen.

Although fresh pollen produced ears with percentages of waxy seeds ranging from 33.1 to 55.9, there was no tendency for these differences to be maintained when stored pollen was used. The correlation between the percentage of waxy seeds resulting from the use of fresh pollen and that obtained from stored pollen was only  $0.16 \pm 0.13$ , indicating that the conditions of storage obliterated any differences between fresh samples.

If the storage operated to bring the functioning waxy grains up to equality with the horny grains, a negative correlation should be found between the percentage of waxy seeds on the ears resulting from fresh pollen and the difference between this percentage and that obtained

from the use of stored pollen. This would follow since the fresh samples giving the lowest percentage should be (under the premise) the samples that produced the largest differences. The correlation is found to be  $-0.302$ , and although the coefficient has no statistical significance,  $P$  lying between  $0.1$  and  $0.2$ , it does indicate that the deficient percentages obtained from fresh pollen tend to be corrected in the stored pollen.

At Arlington, on August 8, a series of exposures to sunlight was made covering a period of 4 hours. The strains used were identical with those grown at Lanham. Only nine pairs of ears were obtained, and these showed no evidence of alteration in the proportion of waxy to horny pollen grains when exposed to sunlight. None of the 10 differences is statistically significant.

This result may be compared with the results obtained at Lanham on the same day, where 13 of the 26 differences were significant, 10 being in the direction of a higher percentage of waxy seeds from treated pollen. Since the exposure at Arlington was for 4 hours and the shortest exposure at Lanham was 6 hours and 50 minutes, the lack of agreement between the two places may be due to this difference in period of exposure.

On the same day at Arlington a second group of pollinations was made, in which the fresh pollen was used on homozygous waxy plants and the stored pollen was used to self-fertilize the plants that functioned as male parents. This was done because there was a possibility that the pollen might behave differently when used on the stigma of the plant that produced it than when used on sister plants. The fresh pollen proved to have  $47.12 \pm 0.57$  percent waxy grains. The expected percentage of waxy seeds when this pollen is used on plants heterozygous for waxy is  $23.6$ . The self-pollinated ears resulting from the use of stored pollen had  $23.5 \pm 0.4$  percent of the seeds waxy. This agreement with the percentage expected from the known proportion of waxy to horny pollen grains in the fresh pollen is added evidence that 4 hours' exposure to sunlight failed to alter the relative effectiveness of waxy pollen grains.

In nine instances stored pollen was used both in backcrosses and in self-pollinations, permitting direct comparisons of the two types of pollination. The mean difference between the self-pollinated and the backcrossed ears, after doubling the percentages in the groups of self-pollinated ears to make them comparable with those obtained from backcrossing, is found to be  $-1.4 \pm 1.7$ , showing that the results of pollen storage are not affected by the nature of the pollination, i. e. whether selfed or backcrossed.

There was no correlation between the percentage of waxy seeds on the backcrossed and on the selfed ears where stored pollen was applied ( $\rho = -0.221 \pm 0.224$ ). With only nine comparisons, however, the correlation must be very close for statistical significance.

At Arlington, Va., in 1929, an attempt was made to accumulate the data necessary to determine the change in proportion of functioning waxy pollen grains with increase in time of storage.

A bulk sample of pollen was collected, subdivided into small containers, some of these samples were used immediately for fresh controls and the remainder stored in the sun. Each hour a certain number of exposed samples were applied to ears of homozygous plants. It

was found necessary to make two collections of pollen, and unfortunately these two lots did not react alike to storage.

The summarized data are shown in table 6. As will be seen, the pollen from the first lot showed a change in the percentage of waxy seeds after 2 hours' exposure to sunlight and an increased alteration with 3 hours of storage, which was the final period for the first bulk sample of pollen. The second lot, although showing an increase in percentage of waxy with the passage of time up to 6 hours, fell far short of the change observed in 3 hours in the first lot. Since both lots of pollen had identical exposures for the first 3 hours, the discrepancy between them cannot be charged to different amounts of radiation. Both lots of pollen were alike in their behavior when used fresh, and since they consisted of bulk samples from sister plants there can be no question of plant sensitivity. Further, since the pollen was all subdivided into small lots, each subsample containing enough pollen for two ears, it hardly can be maintained that variation in the quantity of pollen was a factor. Although it is probably true that the samples contained unequal amounts of pollen, it is unlikely that all the subsamples of one collection contained more or less pollen than all those of the other collection.

TABLE 6.—Percentage of waxy seeds obtained from fresh and from sun-treated pollen, Arlington, Va., August 6, 1929

Treatment of pollen	Ears	Total seeds	Waxy seeds	Treatment of pollen	Ears	Total seeds	Waxy seeds
Fresh (first collection).....	Number 6	Number 2, 473	Percent 45.8±1.4	Fresh (second collection).....	Number 11	Number 4, 775	Percent 43.7±.9
Hours in sun.....				Hours in sun.....			
1.....	13	5, 258	45.5±.7	4.....	14	6, 479	44.1±.8
2.....	10	3, 223	51.6±1.7	5.....	10	5, 143	47.0±1.0
3.....	11	3, 196	63.0±1.4	6.....	12	4, 142	52.7±1.4

At Sacaton, Ariz., in 1929, two lots of ears were self-pollinated, one lot with pollen collected and applied between 7:30 and 8 a. m., the other with pollen collected during this period and exposed to direct sunlight through one thickness of glassine paper for 8 hours and 45 minutes. Since the ears were self-pollinated, identical plants are not involved, but the number of plants was such that an average sample was assured. The ears obtained from the use of fresh pollen had a mean of  $20.9 \pm 0.5$  percent waxy seeds and those resulting from the use of stored pollen had  $23.0 \pm 1.1$  percent waxy. The difference between the two groups is not significant. There is a significant negative correlation between the number of seeds and the percentage of waxy in the group of ears obtained from the use of stored pollen,  $\rho$  equaling  $-0.584$ , and  $P$  being less than 0.01. In the fresh pollinations the correlation coefficient is of the same sign but is not significant ( $\rho = -0.291$ ).

There is no difference between the two populations in mean seed number, the stored group having a mean of 157 and the fresh group a mean of 150 seeds per ear. The fact that a higher percentage of waxy seeds is found when the number of seeds is small might be taken as an indication that the slower rate of germination of waxy pollen grains accounted for the observed correlation in the group of ears from stored pollen. However, this relationship would be expected



as well in the group of ears from fresh pollen, on the premise that scant quantities of pollen offer no opportunity for competition between the slow- and fast-germinating sorts of pollen grains.

The fact that no such relationship between number of seeds and percentage of waxy is found in the fresh group as is revealed in the stored group indicates that the unfavorable conditions of storage in sunlight operate to destroy horny pollen grains more rapidly than waxy grains.

At Lanham in 1932 a series of exposures was made in direct sunlight for a period of one-half hour. Half-hour exposures were made on three different days, the radiation as measured with the photronic cell ranging from 2,000 to 12,500 foot-candles. On none of the 3 days was the treatment effective, and the mean change for the combined series was an increase from treated pollen of  $1.08 \pm 0.41$  percent waxy.

On one of the days, treatments of 15, 30, and 45 minutes were given. Several of these were successful in all categories. The data are presented in table 7. In only one instance (sample 3005, exposed for 45 minutes) was there evidence of a significant change in the proportion of functioning waxy pollen grains. This sample shows a difference of  $16.0 \pm 2.2$  percent between the effectiveness of the stored pollen and that of the fresh pollen, the stored pollen producing the higher percentage of waxy seeds.

Longer exposures were tried in this year, but only three pairs were successful. These were given 5 hours at rather low candlepower (180 to 7,000 foot-candles), with temperatures ranging from  $28^{\circ}$  to  $41^{\circ}$  C. All three samples show very great differences in the percentage of waxy seeds between ears resulting from the use of stored pollen and those from the use of pollen exposed to sunlight. The mean difference in percentage of waxy seeds for these three ears is  $42.9 \pm 3.3$ , the pollen stored in sunlight giving the highest percentage.

TABLE 7.—Percentage of waxy seeds obtained from fresh and from sun-treated pollen<sup>1</sup>

Pollen sample no.	Seeds resulting from pollen used after indicated treatment							
	Fresh		In sun 15 minutes		In sun 30 minutes		In sun 45 minutes	
	Total	Waxy	Total	Waxy	Total	Waxy	Total	Waxy
	Number	Percent	Number	Percent	Number	Percent	Number	Percent
2437.....	1,347	38.6 $\pm$ 1.1	1,223	40.2 $\pm$ 0.9	999	45.2 $\pm$ 1.1	676	47.9 $\pm$ 1.3
2486.....	2,318	44.6 $\pm$ .7	1,378	48.3 $\pm$ .9	892	51.0 $\pm$ 1.1	555	48.3 $\pm$ 1.4
2816.....	1,962	45.3 $\pm$ .8	942	41.5 $\pm$ 1.1	942	43.9 $\pm$ 1.1	598	47.5 $\pm$ 1.3
2825.....	1,418	45.3 $\pm$ .9	1,303	46.9 $\pm$ .9	936	43.6 $\pm$ 1.1	1,278	47.2 $\pm$ .9
3005.....	2,296	45.6 $\pm$ .7	1,245	42.5 $\pm$ .9	945	48.8 $\pm$ 1.1	250	61.6 $\pm$ 2.1
3007.....	2,169	48.9 $\pm$ .7	1,285	45.6 $\pm$ .9	1,240	44.3 $\pm$ 1.0	885	48.0 $\pm$ 1.1
3105.....	582	44.7 $\pm$ 1.4	1,100	45.9 $\pm$ 1.0	767	47.2 $\pm$ 1.2	7	85.7 $\pm$ .9

<sup>1</sup> The illumination varied from 4,400 to 6,400 foot-candles, and the temperature ranged from  $37^{\circ}$  to  $50^{\circ}$  C.

#### STORAGE AT VARIOUS TEMPERATURES AND IN OXYGEN

In order to differentiate further between temperature and light it would be desirable to have light exposures at low temperatures and heat exposures in the dark. The latter, of course, are easy to provide, but the former can be obtained only imperfectly. It is not possible to vary the illumination without a concomitant alteration

in the temperature, except by means of quartz cells and flowing water. Even with this device, which restricts the number of pollen samples handled and increases the time interval for equal energy intake, the quality of radiation is somewhat altered. Not only is it difficult to provide light exposures without heat but it is equally difficult to determine the temperature of pollen grains exposed to light. Just what proportion of the radiant energy is transformed into heat when absorbed by the opaque yellow pollen grains and how much of this heat is counteracted by evaporation it is now impossible to measure. Crude attempts have been made to estimate pollen temperatures by means of pollen-covered mercury thermometers, but the information derived from this method is admittedly inaccurate.

The tests show that such thermometers exposed to sunlight may exceed air temperatures by as much as 25° C., the amount depending on the brightness of the day, air movement, and air temperature, to say nothing of the thickness of the pollen coating and its moisture content.

If the observed increase in proportion of waxy to horny seeds following the use of pollen stored in direct sunlight is simply the result of high temperatures, then pollen stored at temperatures of 35° to 45° C. in the dark should produce similar changes in the percentage of waxy seeds.

To test this point a vertical oven was constructed with a light bulb at the bottom as the source of heat. This oven, with four wire shelves and a damper control, proved to give very constant temperatures. The four stages provided temperatures of 31°, 32°, 35°, and 45° C., which could be held within a range of 1° with infrequent attention to the damper.

Pollen collected from 50 heterozygous plants was sieved to remove anthers and thoroughly mixed. This sample was then divided into subsamples, which were placed in small glassine bags, each subsample having enough pollen to serve 2 years. The glassine bags were then numbered, and the pollen from one was used immediately to pollinate two ears of homozygous waxy plants. The silks of these ears had been cut back 24 hours previously to provide a uniform brush about 1 inch long. The bags of pollen to be treated were subjected to the four oven temperatures for 3½ hours, after which the pollen was applied to ears in the same progeny as the ears pollinated with the fresh pollen. Because of the distance from the field to the laboratory and the time consumed in making the pollinations, the period elapsing between the time the treated pollen was collected and the final application was 5½ hours. Each of the subsamples was used on 2 ears, except that in a few cases, where one of the ears had but few silks, three ears were pollinated from a single subsample. The results are summarized in table 8.

It will be seen at once that the treatments, far from increasing the percentage of waxy seeds, actually reduced it. The control lot deviated slightly but not significantly below equality, but without exception all the heat treatments tried in this experiment resulted in significant reductions in the percentage of waxy seeds.

TABLE 8.—Percentage of waxy seeds obtained from pollen subjected to various temperatures for  $3\frac{1}{2}$  hours

Treatment (° C )	Ears	Total seeds	Waxy seeds
	<i>Number</i>	<i>Number</i>	<i>Percent</i>
Control <sup>1</sup> .....	3	846	47.87±1.15
31.....	6	2,310	39.44±1.05
32.....	8	1,453	38.13±1.17
35.....	11	3,076	40.08±.85
45.....	9	2,469	39.69±1.57

<sup>1</sup> Pollen applied immediately after subsampling

A second experiment was conducted with pollen handled in the same fashion. In this experiment pollen was collected from 50 plants and thoroughly mixed. Subsamples were then poured into small glassine bags, which were assigned consecutive numbers. These subsamples were divided into three groups. One group was stored for  $2\frac{1}{2}$  hours at  $37^{\circ}$  C., another was stored for the same length of time at  $10^{\circ}$ , and the third was held in an atmosphere of oxygen drawn into jars through sulphuric acid. All three treatments gave very low percentages of waxy seeds, the mean percentages being  $32.49 \pm 0.49$ ,  $31.18 \pm 0.53$ , and  $37.73 \pm 0.45$ , respectively. The results of the two temperature treatments were nearly alike in the percentage of waxy seeds, indicating complete absence of temperature effect and pointing clearly to some other disturbing factor.

These ears are the first in which a reduction in the percentage of waxy seed was observed to follow the use of stored pollen, but other lots were encountered subsequently and similar reductions have been reported by Brink (2). Further it should be noted that all pollen produced at the Arlington Experiment Farm in 1928 and subjected to various treatments gave similar reductions in the percentage of waxy seeds. Although the experiment affords no proof that high temperatures offer the explanation for the observed effect of storage of pollen in the sunlight, it must be borne in mind that the technique followed in handling the pollen samples differed radically from that previously employed. In fact the general reductions in the percentage of waxy seeds observed in all pollen samples handled in bulk at Arlington raises the question as to whether the results are not directly ascribable to this method of handling the pollen.

In addition to the oxygen series already discussed, storage in oxygen was tried on 3 other days. As it happens, no other ears are available for comparison on 2 of these days, as all other treatments tried, such as high temperatures, carbon dioxide, etc., proved lethal. However, a series of 10 ears resulted from fresh pollen used on 1 day (Aug. 4). These 10 ears had a mean of  $46.24 \pm 0.78$  percent waxy seeds. The summarized data for the oxygen treatments are given in table 9.

All four groups of ears resulting from pollen stored in oxygen had less than half of the seeds waxy, and the deficiency became greater as the season advanced. No significance is attached to this relationship, as numerous attempts to discover a seasonal trend in the waxy-horny ratio in many progenies have always resulted in failure. However, Chao (4), working with the glutinous gene in rice (which

corresponds in effect very closely with the waxy gene in maize), found evidence of a seasonal trend in the survival of pollen grains bearing this gene.

TABLE 9.—Percentage of waxy seeds obtained from pollen stored in oxygen for  $4\frac{1}{2}$  hours

Date	Ears	Total seeds	Waxy seeds
	Number	Number	Percent
July 31.....	5	1,727	48.52±1.23
Aug. 1.....	7	2,345	46.31±1.31
Aug. 4.....	4	137	40.88±1.30
Aug. 7.....	22	9,449	37.73±.45

When the mean percentage of waxy seeds obtained from the use of fresh pollen ( $46.24 \pm 0.78$ ) was compared with that obtained from pollen stored in oxygen ( $40.88 \pm 1.30$ ) on August 4, the difference was found to be  $5.36 \pm 1.52$ , the oxygen-treated pollen giving the lower percentage.

At Lanham, Md., in 1928, bulk samples of pollen were collected from heterozygous plants, but, instead of combining the pollen of 50 plants, only 10 plants were used and several collections were made. Numerous exposures to sunlight were tried, but in every case this treatment killed all the pollen.

One experiment involved dividing the bulk pollen into three subsamples, applying one subsample fresh to several ears, exposing a similar quantity to direct sunlight for several hours, and placing the third lot in an oven. Five bulk samples, collected at five different hours in 1 day, were treated in this manner. Not a single lot exposed to sunlight produced seed, but the oven samples were more successful. These are compared with the controls in table 10. The longest period of treatment corresponds with the earliest time of collection, since all treated samples were removed from the oven at the same time.

TABLE 10.—Percentage of waxy seeds obtained from fresh pollen and from duplicate pollen samples held in the dark for various periods and at various temperatures

Sample no.	Hour of collection	Treatment		Ears	Total seeds	Waxy seeds
		Temperature	Duration			
	A. m.	° C.	Hours	Number	Number	Percent
1.....	8 00	( <sup>1</sup> ) 35	8½	7	1,729	50.64±1.03
		( <sup>1</sup> ) 26	8	5	145	71.04±3.49
2.....	8 30	( <sup>1</sup> ) 28	8	5	1,398	48.64±1.51
		( <sup>1</sup> ) 26	8	5	700	46.15±1.41
3.....	9 30	( <sup>1</sup> ) 28	7	8	1,076	46.40±.82
		( <sup>1</sup> ) 26	7	8	1,980	49.04±1.06
4.....	10 15	( <sup>1</sup> ) 26	6½	10	2,638	47.42±1.01
		( <sup>1</sup> ) 26	6½	13	3,105	49.08±.80
5.....	10 50	( <sup>1</sup> ) 35	5¾	1	5,491	48.42±.37
		( <sup>1</sup> ) 35	5¾	1	168	53.00±2.50
		( <sup>1</sup> ) 35	5¾	4	1,525	49.51±.95

<sup>1</sup> Pollen applied fresh.

It will be noted that, with the exception of the 8½-hour treatment at 35° C., all treatments resulted in a lower percentage of waxy seeds than their corresponding controls. The group of five ears resulting from the 8½-hour treatment and having a mean percentage of  $71.0 \pm 3.5$  waxy seeds was consistent, although, as the total (145) shows, the ears had very few seeds. So far as known, there was no difference in these samples other than the time at which the pollen was collected and the length of the storage period. However, from the fact that the pollen collected at 10:50 a. m. and stored for 5¼ hours at 35° gave no evidence of an increased survival of waxy pollen grains, it is highly doubtful that the change observed in lot 1 after 2¼ more hours of storage could have resulted from the additional time unless a definite threshold reaction be assumed. It seems more likely that the pollen collected at the earlier period was in some manner more sensitive to the conditions imposed and that the waxy grains were slightly more able to resist the unfavorable treatment. Certainly the number of seeds obtained from this lot of treated pollen was very much less than that obtained from lot 5.

In the years 1931 and 1932 all pollinations were made on the individual plant basis. Each sample of pollen from a heterozygous plant was subdivided and one lot was used fresh as a control. Various periods of exposure and several stages of heat treatment were tried. The results are summarized in table 11. When the array of mean differences is examined, it is seen that the percentages of waxy seeds from stored pollen range from a deficiency of  $3.0 \pm 0.9$  to an excess of  $17.3 \pm 4.6$ . The duration of the treatments ranged from 15 minutes to 2 hours, and the temperature to which the several samples were subjected ranged from 40° to 50° C. In attempting to derive order from this series, it became apparent that the closest relationship is that between the length of the exposure period and the degree of alteration in the proportion of functioning waxy pollen. The coefficients of correlation of ranks calculated for these variables are as follows: Between the difference in the percentage of waxy seeds and duration of exposure  $\rho = 0.614$ ; between the difference and temperature  $\rho = -0.25$ ; and between duration and temperature  $\rho = -0.7$ . The last-named coefficient, of course, results largely from design, since for the most part the pollen samples were kept at the highest temperatures for the shorter periods. With only 9 points, these coefficients can be only suggestive.

There can be no question but that the series of ears resulting from the use of pollen stored in the dark for 2 hours at 41° C. in 1932 had significantly higher percentages of waxy seeds than the ears pollinated with duplicate samples of fresh pollen. However, even in this series of nine comparisons there were three where no effect of storage was obtained. In the other groupings there are scattered samples where unquestionable alterations have occurred, especially in the series of 12 ears held on July 30, 1932, for 1½ hours at 47°. The series as a whole is so variable that the mean change is hardly significant.

In addition to the scattered samples showing increased effectiveness of waxy pollen grains following storage, there are several cases where the treatment seems to have acted unfavorably on the waxy pollen.

TABLE 11.—Percentage of waxy seeds from fresh pollen and from pollen stored for various periods and at various temperatures<sup>1</sup>

Date	Treatment		Paired ears	Total seeds from indicated pollen		Difference in waxy seeds from fresh and from stored pollen
	Duration	Temperature		Fresh	Stored	
	<i>Minutes</i>	<i>° C</i>	<i>Number</i>	<i>Number</i>	<i>Number</i>	<i>Percent</i>
1931						
July 28	15	42	9	6,316	5,049	3.0±0.9
July 29	30	49	5	2,261	1,316	-3.6±3.0
July 30	30	44	4	2,425	1,270	7±2.2
Aug. 1	30	40	2	1,121	1,857	1.4±1.0
Aug. 2	20	50	7	3,999	4,062	1.1±2.3
1932						
July 28	90	44	2	1,969	2,128	1.1±2.1
July 30	90	47	12	16,613	13,724	-7.8±2.6
Aug. 3	75	41	8	10,430	10,031	-3±.7
Do	120	41	9	11,660	8,184	-17.3±4.6

<sup>1</sup> Pollinations were made with collections from individual plants, each pollen parent furnishing a fresh and a treated sample.

One other series of comparisons of pollen treated in the dark remains to be discussed. In this last series individual plant samples were subdivided into two lots. One lot was stored at 10° C, the other at 25° for periods of 7 hours and 10 minutes to 7 hours and 30 minutes, both being in the dark. The pollen stored at 25° gave a slightly higher percentage of waxy seeds than that stored at 10° but the difference (2.6±.9) is not significant. Both samples gave mean percentages of waxy seeds slightly above 50, which is unusual.

#### STORAGE UNDER ARTIFICIAL LIGHT

For experiments designed to determine the effect of light on the survival of pollen grains, the sun constitutes a capricious and an uncertain source of illumination since sunlight varies in intensity with each passing cloud. Even on bright clear days the intensity of radiation varies, and exposures to uniform amounts of radiation are not often obtainable. The chief advantages of sunlight lie in the amount of energy delivered and in the area covered, which makes possible the exposure of large numbers of samples. These advantages are offset by the difficulty in estimating the amount of radiation delivered over a given period. The development of the photronic cell has provided a means of rapidly measuring changes in radiation, but where the radiation is varying during an exposure period even frequent readings of this instrument permit only a coarse estimate of the amount of radiation. Accordingly a controlled source of illumination was sought.

It was known from the experiments of Brink (2) with ultraviolet and also from the fact that sunlight is effective through glassine paper (having a transmission spectrum similar to that of window glass) that wave lengths in the ultraviolet region were not essential in differentiating waxy from horny pollen grains.

It was desirable that the artificial source of illumination should have an emission spectrum similar to sunlight and that the energy output should be such that long exposures were not required.

Providentially there was introduced at about this time a lamp designed to meet the therapeutic fad of the moment for home sun-tanning. This lamp ingeniously combines the mercury vapor arc with a glowing tungsten filament and when equipped with a Corex glass bulb affords in simple form a fair approximation to sunlight, though somewhat deficient in the middle region of the spectrum. The radiant energy at a distance of 20 cm from the source is roughly 0.1 that of sunlight at the earth's surface on a bright day.

This light was placed in operation in 1930, a year that in the vicinity of Washington, D. C., was notable for an extended drought. Preliminary experiments showed that pollen exposed to this light for one-half hour failed to germinate on corn silks, but shorter exposures offered more hope. The treatments were planned, therefore, on the basis of exposures of 5, 10, and 15 minutes, and in addition some samples were illuminated through a quartz cell carrying a stream of cold water as a means of preventing a rise in temperature.<sup>4</sup>

The rigors of the season played such havoc with the results that few ears were obtained and these had very few seeds each. A single complete set, having enough seeds to carry some statistical weight, represents the results for this year. These data are shown in table 12.

TABLE 12.—Percentage of waxy seeds obtained from subsamples of pollen from a single plant, applied fresh or after exposure for various periods to artificial light of 1,200 foot-candles<sup>1</sup>

Treatment		Total seeds	Waxy seeds
Duration	Temperature		
Minutes	°C.	Number	Percent
(?)	36	202	48.6±2.0
5	38	122	54.9±3.0
10	40	100	64.0±3.2
15	49	177	71.8±2.3
15 <sup>2</sup>	43	135	51.1±2.9

<sup>1</sup> Estimated from known distance between lamp and pollen.

<sup>2</sup> Pollen applied fresh.

<sup>3</sup> Pollen protected from high temperature by quartz water cell.

The pollen used on these five ears was from a single heterozygous plant, and the results show that the factors operating during the exposures to light, in the absence of the quartz water cell, brought about a decided alteration in the proportion of waxy to horny pollen grains functioning in fertilization. The single ear resulting from the use of pollen exposed to light for 15 minutes but protected from high temperature by the quartz cell shows equally clearly that light alone does not affect these two sorts of pollen grains differentially.

The temperature as measured by an exposed mercury-bulb thermometer rose under the quartz water cell from 36° to 43° C., whereas without the protection of the water cell the temperature attained a maximum of 49°. As it has been shown that temperatures of this degree for such short periods are ineffective, the possibility may be entertained that the differential functioning of these two sorts of pollen grains is brought about by the combination of light and heat.

<sup>4</sup> The writer is indebted to Dr. F. S. Brackett, formerly of the Division of Radiation and Organisms, Smithsonian Institution, for suggesting the use of this device and for the cell itself.

Slight support for this hypothesis is derived from a summary of all the seeds obtained from these experiments in 1930.

The high mortality of all pollen in 1930 prevented comparisons of individual pollen samples, but the seeds may be summarized. From the use of fresh pollen, 3,862 seeds were obtained having  $50.0 \pm 0.6$  percent waxy; from pollen treated with artificial light not protected by the quartz water cell, 1,094 seeds were obtained having  $53.0 \pm 1.0$  percent waxy; when the pollen was screened by the quartz water cell, 2,479 seeds were obtained with  $49.1 \pm 0.7$  percent waxy; and when pollen was stored in the dark at temperatures identical with those of the unprotected light series, 260 seeds were obtained having  $50.1 \pm 2.1$  percent waxy. The errors in the above summary are calculated from the total number of seeds and no great significance can be attached to the results. There is thus a slight indication that light plus heat produces an effect not obtained with either factor alone, but the data are far from conclusive.

Since the data obtained in 1930 were inadequate, an extensive series of exposures to artificial light was tried in 1931. These exposures ranged from 5 to 45 minutes, all without the quartz water cell.<sup>5</sup> The results are shown in the first 11 entries in table 13. Not one of the treatments produced a significant alteration in the proportion of waxy to horny pollen functioning in fertilization.

TABLE 13.—Comparison of results obtained from fresh pollen and from pollen exposed for various periods to artificial light of 1,200 foot-candles<sup>1</sup>

Treatment <sup>2</sup>			Paired comparisons	Total seeds from indicated pollen		Difference in waxy seeds from fresh and from treated pollen
Date	Duration	Temperature		Fresh	Treated	
1931			Number	Number	Number	Percent
July 25.....	20	—	5	2,331	2,027	$-0.2 \pm 2.0$
July 26.....	10	—	11	3,975	3,717	$0.0 \pm 1.2$
Do.....	5	—	6	2,798	2,508	$1.5 \pm 1.7$
July 28.....	5	47	6	3,908	2,790	$3.8 \pm 1.7$
Do.....	15	45	7	4,968	5,005	$0.0 \pm 1.4$
July 30.....	20	44	5	2,689	2,463	$-3.6 \pm 2.1$
Do.....	30	46	5	5,705	2,181	$1.7 \pm 1.3$
Aug 1.....	15	39	9	4,888	2,901	$2.5 \pm 1.1$
Do.....	30	42	9	5,209	6,761	$4.6 \pm 1.1$
Do.....	45	43	4	1,514	1,550	$0.2 \pm 1.7$
Aug. 2.....	20	44	5	2,930	1,581	$-2 \pm 2.0$
1932						
July 30.....	<sup>3</sup> 60	37	6	10,130	3,302	$-9.6 \pm 3.1$
Do.....	90	47	5	7,596	25	$-65.0 \pm 3.0$
Aug 3.....	75	40	5	6,840	1,384	$-47.0 \pm 1.9$
Do.....	120	41	6	8,224	157	$-58.8 \pm 1.2$

<sup>1</sup> Estimated from known distance between lamp and pollen. At this distance the pollen was exposed to 1,200 foot-candles in 1932, when the illumination was measured by the photronic cell.

<sup>2</sup> Pollinations were made from collections from individual plants, each pollen parent furnishing a fresh and a treated sample.

<sup>3</sup> At 800 foot-candles.

A single experiment was arranged whereby the pollen samples were exposed at different distances from the source of light, all being exposed at the same time and for the same period. The distances were so arranged that the uppermost sample received four times the radiant

<sup>4</sup> The photronic cell was not available in this year, but the following year it was found that at the distance that separated them from the lamp the pollen grains were subjected to light of an intensity of 1,200 foot-candles.



energy of the lowest sample. The comparison involved fresh pollen and pollen exposed for 20 minutes at distances of 32 cm, 24 cm, and 16 cm from the light. The following percentages of waxy seeds were obtained: From the fresh pollen,  $45.6 \pm 0.7$ ; from the pollen exposed at 32 cm,  $43.2 \pm 0.6$ ; at 24 cm,  $44.1 \pm 0.7$ ; and at 16 cm,  $45.3 \pm 0.8$ . Clearly for the time involved (20 minutes) the light produced no effect on the proportions of the two sorts of pollen grains, nor is there any evidence that the ratio varied with the energy received.

In view of the conflicting results in these experiments with pollen ratios, further experiments were conducted in 1932. The summarized results are shown in the last four entries in table 13. Exposures as short as 1 hour with an illumination of 800 foot-candles, as measured with the photronic cell, produced a marked differential in the survival of the two sorts of pollen grains in certain samples. When the light intensity was raised to 1,200 foot-candles and the exposure time was extended beyond 1 hour, the treatment was almost completely lethal, but of the pollen grains that survived by far the largest proportion bore the waxy gene. With one exception (the  $1\frac{1}{2}$ -hour exposure at 1,200 foot-candles), the temperatures of the treatments were not so high as those of 1931, but the difference in effect between the 2 years is striking.

A direct comparison of the effect of artificial illumination and of treatment in the dark at the same temperature is given in table 14. The ears in each comparison are from identical lots of pollen collected from individual male parents. The pollen samples stood side by side during treatment, the only difference between them being that the dark series were in covered pill boxes. The number of seeds in the light series is unfortunately small, and indeed only two of the ears in this series have any significance by themselves. In the aggregate, however, they emphasize the greater effect obtained with relatively high temperatures in conjunction with light as compared with the effect of similar temperatures in the dark.

TABLE 14.—Percentage of waxy seeds obtained from pollen subjected to artificial illumination of 1,200 foot-candles and from duplicate samples of pollen stored in the dark

Treatment		Pollen sample	Seeds from pollen exposed to—			
			Dark		Light	
Duration	Temperature <sup>1</sup>		Total	Waxy	Total	Waxy
Hours	° C.	No.	Number	Percent	Number	Percent
$1\frac{1}{2}$	47	2339	2,306	$50.0 \pm 0.7$	3	100
		2353	1,008	$68.1 \pm 1.0$	7	100
		2437	1,272	$37.8 \pm .9$	122	$66.4 \pm 2.9$
		2461	1,936	$47.0 \pm .8$	4	100
		2486	161	$84.5 \pm 1.9$	7	100
		3007	58	$81.5 \pm 3.2$	6	$83.3 \pm 10.2$
		2816	450	$92.3 \pm 1.2$	16	100
		2694	672	$48.1 \pm .7$	116	100
2	41	2658	2,020	$48.0 \pm .8$	8	100
		2843	1,862	$54.5 \pm .8$	10	$90 \pm 6.4$
		3218	422	$49.1 \pm 1.2$	6	100
2	47	3629	43	$53.5 \pm 5.1$	5	100

<sup>1</sup> The temperatures given are the maximum attained during treatment.

## INDIVIDUAL PLANT RESPONSES TO POLLEN TREATMENTS

The individuality of the reactions of the various pollen samples to the dark treatment has already been pointed out; a more detailed discussion follows:

As an illustration of the variability of reaction of different samples of pollen, the data presented in table 15 have been assembled. The pollen was collected from four sister plants at 8 a. m., July 30, 1932, and subjected to various treatments. These four plants were chosen for this presentation because the treatments were alike and reasonably successful in all categories. It will be observed that (1) only the pollen produced by plant 3005 showed a reaction to sunlight, (2) only the pollen from plant 2825 failed to react to 47° C. in the dark for 1½ hours, (3) the pollen of plants 2486 and 3005 failed to show any effect of storage for 24 hours, and (4) the pollen of plant 3007 failed to respond to artificial illumination of 1 hour's duration.

The pollen from all plants responded to some treatments, and it is not possible to conclude that the pollen of one plant is more or less sensitive than that of another. Apparently the condition of the pollen determines its response to a particular treatment. Thus pollen from plant 3005 reacted to sunlight, artificial light, and high temperature in the dark, but storage for 24 hours failed to bring about a change. On the other hand, pollen from plant 2825 showed only a relatively slight effect when subjected to artificial illumination but a pronounced reaction to prolonged storage.

Because of their individuality, these responses give little insight into the nature of the reactions causing the differential survival of the two sorts of pollen, but they help to explain the variability encountered in arrays of ears obtained from the use of treated pollen.

TABLE 15.—Percentage of waxy seeds from pollen collected from 4 plants at the same time, July 30, 1932, and subjected to light and darkness for various periods and at different temperatures

Treatment				Seeds from pollen collected from plant no. —							
Exposure	Duration	Light intensity	Temperature <sup>1</sup>	2825		2486		3005		3007	
				Total	Waxy	Total	Waxy	Total	Waxy	Total	Waxy
	Minutes	Foot-candles	° C.	Number	Percent	Number	Percent	Number	Percent	Number	Percent
Fresh.....				1,418	45.3±0.9	2,318	44.6±0.7	2,296	45.6±0.7	2,169	48.9±0.7
Sun.....	15	4,000	42	1,303	46.9±.9	1,378	48.3±.9	1,245	42.5±.9	1,285	45.6±.9
Do.....	30	4,400	42	936	43.6±1.1	892	51.0±1.1	945	48.8±1.1	1,240	44.3±1.0
Do.....	45	4,400	42	1,278	47.2±.9	555	48.3±1.4	250	61.6±2.1	885	48.0±1.1
Light.....	Hours										
Do.....	1	800	37	1,347	53.3±.9	18	88.9±5.2	755	59.3±1.2	1,006	51.7±.8
Do.....	1½	1,200	47			7	100.0			6	83.3±10.3
Dark.....	1		37					1,353	47.2±.9	1,005	45.9±1.1
Do.....	1½		47	1,179	43.1±1.0	161	84.5±1.9	199	73.9±2.1	58	84.5±3.2
Do.....	24		26	253	74.3±1.9	277	44.8±2.0	708	45.9±1.3	1,155	64.0±1.0

<sup>1</sup> Maximum attained during treatment

## DURATION OF STORAGE

## SEVENTEEN HOURS

By collecting pollen in the afternoon, using one lot immediately as a fresh control, and applying the remainder the following morning, a storage period of 17 hours was obtained, during which the pollen was kept in covered containers at room temperature.

In this experiment pollen was collected from individual plants at Arlington, Va., at 4 p. m., August 6, 1929. The stored pollen was applied at 9:30 a. m. the following morning, giving approximately 17 hours of storage. In 19 samples the pollinations were successful in providing ears from both the fresh and the stored pollen. The ears obtained from the fresh pollen had the highest percentages of waxy seeds, but the difference of  $2.62 \pm 1.15$  is not significant.

Although there were only 19 successful paired pollinations, 25 ears were obtained from fresh pollen and 26 ears resulted from the use of stored pollen. When these two groups were examined from the standpoint of their female parents there was seen to be strong evidence of the influence of the female parent on the percentage of waxy seeds. The waxy plants used as female parents in this experiment were in two progenies. The plants in one group were designated by numbers between 1200 and 1300; in the other group, by numbers between 500 and 700.

Fresh pollen applied to the plants numbered between 1,200 and 1,300 produced 49.1 percent waxy seeds. Similar pollen applied to plants of the other progeny produced a mean percentage of 47.7 waxy seeds. The two groups are in fair agreement,  $\chi^2$  equaling 2.06 ( $P=0.147$ ) and the mean of the two being 48.4. However, when stored pollen was applied to plants numbered between 1,200 and 1,300, the resulting seed was but 41.96 percent waxy, whereas similarly treated pollen used on plants numbered between 500 and 700 gave ears with a mean of 51.51 percent waxy seeds. The plants furnishing the pollen were not sibs of either progeny used as female parents. The  $\chi^2$  of the difference between these two progenies is 7.05 ( $P=0.008$ ).

In one case storage decreased the effective proportion of waxy pollen grains, whereas in the other case the proportion of these pollen grains was increased. Clearly there is an effect of the female parent on the behavior of stored pollen. No such effect can be demonstrated for the fresh pollen; in fact, the progeny that gave the highest percentage of waxy seeds from the use of stored pollen gave the lowest percentage when fresh pollen was used. The difference between the two progenies in their reaction to stored pollen as compared with fresh pollen is  $10.99 \pm 2.21$ . The data are given in table 16.

TABLE 16.—*Effect of female parent on percentage of waxy seeds from fresh and from stored pollen*

Female parent progeny no.	Waxy seeds from indicated pollen		Difference (fresh—stored)
	Fresh	Stored	
	Percent	Percent	Percent
1200.....	49 14±0.79	41.96±1.02	7.18±1.29
500.....	47 70±1.34	51.51±1.20	-3 81±1.80
Difference.....	1 44±1.55	-9 55±1.57	10.99±2.21

At Sacaton, Ariz., 1929, a short series of plants was self-pollinated, one half with pollen collected and applied in the afternoon, the other half with pollen stored overnight and applied the following morning. The period of storage was 16 hours and 5 minutes and, judging from the few ears and small number of seeds, the lethal limit was closely approximated. The ears obtained from the use of fresh pollen had  $18.9 \pm 0.6$  percent waxy seeds, those obtained from stored pollen had  $19.3 \pm 0.8$  percent waxy seeds. The difference is not significant. Both lots of ears have much lower percentages of waxy seeds than is customary in strains where no disturbing factor such as the *su* gene is present.

#### TWENTY-FOUR HOURS

In 1929, 1931, and 1932 there were a number of instances where pollen was stored in the dark (in covered pill boxes) for 24 hours. In these cases the pollen was collected in the morning and applied the following morning. In 1932 many of these long-period exposures were successful, whereas in other years the majority were failures. The data are presented in table 17. The results are very erratic, with the possible exception of the pollinations made in 1931, where there was a consistent and significant decline in the effective proportion of waxy to horny pollen grains in the stored pollen as compared with the fresh pollen.

TABLE 17.—Percentage of waxy seeds obtained from fresh pollen and from pollen stored for 24 hours in the dark at air temperature

Place	Date	Pollen sample no.	Seeds from indicated pollen				Difference in waxy seed from fresh and from stored pollen	
			Fresh		Stored			
			Total	Waxy	Total	Waxy		
Arlington.....	Aug. 1-2, 1929.....	{	409	267	47.2±2.1	55	100.0	-52.8±2.1
			434	370	56.2±1.7	18	38.9±7.8	17.3±7.9
			701	553	56.2±1.4	45	95.6±2.1	-39.4±2.5
			Av.					-44.9±6.7
		Lanham.....	Aug. 1-2, 1931.....	{	609	376	44.4±1.7	221
680	421				46.8±1.6	220	36.4±2.2	10.4±2.7
1857	482				43.6±1.5	167	32.9±2.5	10.7±2.9
1967	107				61.7±3.2	278	44.2±2.0	17.5±3.8
1969	543				47.0±1.4	331	43.2±1.8	3.8±2.3
Av.								8.5±1.4
Do.....	July 30-31, 1932.....			{	2339	1,203	43.7±1.0	534
		2340	1,378		45.5±.9	410	43.2±1.7	2.3±1.9
		2353	1,055		42.5±1.0	584	46.9±1.4	-4.4±1.7
		2448	1,115		46.1±1.0	289	43.3±2.0	2.8±2.2
		2460	1,006		41.4±1.1	252	42.5±2.1	-1.1±2.4
		2461	1,184		43.0±1.0	385	41.8±1.7	1.2±2.0
		2486	2,318		44.6±.7	277	44.8±2.0	-7.2±1.1
		2513	851		47.4±1.2	323	78.6±1.5	-31.2±1.9
		2542	1,168		46.1±1.0	455	39.8±1.6	6.3±1.8
		2638	495		40.4±1.5	682	42.1±1.3	-1.7±2.0
		2825	1,418		45.3±.9	253	74.3±1.9	-29.0±2.1
		3005	2,296		45.6±.7	708	45.9±1.3	-3.1±1.4
		3007	2,169		48.9±.7	1,155	64.0±1.0	-15.1±1.4
		Av.					-6.1±2.2	

In 1929 and 1932 there were 5 large and unquestionable increases in the percentage of waxy seeds following 24 hours of storage, 3 of

these occurring in 1932. No explanation can be offered as to why these 3 samples behaved so differently from the remaining 10, other than the possible effect of slight differences in quantity of pollen. All of these samples had duplicates that were stored for 1½ hours in the dark at a temperature that reached a maximum of 47° C. Two of these heat-treated duplicates (samples 2448 and 2513) failed to function when used to pollinate waxy plants.

The fact that the heat treatment proved lethal might be urged as indicating a greater sensitivity for these two samples than for the remainder, but 24 hours of storage produced only a negligible change in the proportion of the two sorts of pollen grains in sample 2448, whereas sample 2513 showed a very great change.

Aside from the samples that failed to produce ears, sample 2460 had a period of storage in the sun for 1 hour and a duplicate sample had a covered exposure in the sun designed to equalize temperature effects. The exposure to direct sunlight proved lethal, but the covered sample produced 10 ears showing no alteration in the proportion of the two sorts of pollen grains, the temperature in this case having attained a maximum of 50° C. All the other samples, with the exception of samples 2340, 2353, and 2825, gave significant increases when stored for 1½ hours in the dark at 47°. Apparently the exceptions, 2340, 2353, and 2825, were not sensitive to the treatment, yet sample 2825, when stored for 24 hours, showed a large increase in the functioning proportion of waxy to horny pollen grains.

These 24-hour periods of storage are of interest chiefly in showing that in some instances the processes that effect the differential functioning of these two sorts of pollen grains take place with the passage of time in the absence of light and at ordinary temperatures.

#### EFFECT OF POLLEN STORAGE ON PERCENTAGE OF WAXY SEEDS IN BUTTS AND TIPS

If certain storage conditions gradually reduce the vitality of the pollen grains so that the tubes of weakened grains grow more slowly than those of fully vital grains and if this reduction in vitality takes place faster in horny than in waxy grains, then the proportion of waxy to horny seeds in the butts of ears pollinated with stored pollen should be higher than that found in the tips of the same ears.

In bulk populations where classifications were made separately for the butts and tips, it was found that ears resulting from fresh pollen had  $43.7 \pm 0.6$  percent waxy seeds and that ears resulting from pollen stored in the light for 7½ hours had  $48.4 \pm 0.8$  percent waxy seeds, a difference of  $4.7 \pm 1.0$  due to storage. The increase is not great but it is significant and of sufficient magnitude to show an effect of treatment. Unfortunately, when these pollen samples were treated there was no direct sunlight, the day being cloudy and overcast, with high humidity. This may be the explanation of the slight increase in effectiveness of waxy pollen grains as well as of the unusual number of successful pollinations obtained with stored pollen. Although the observed alteration in the effective proportion of the two sorts of pollen grains is low, certain pertinent information may be derived from a comparison of the percentage of waxy seeds in the butts with that in the tips of the ears.

In a group of 56 ears resulting from the use of stored pollen, the difference between the percentage of waxy seeds in the butts and that

in the tips was  $3.1 \pm 0.4$  percent. In a group of 31 ears resulting from the use of fresh pollen, the difference between the percentage of waxy seeds in the butts and that in the tips was  $0.7 \pm 0.5$ . In both cases the butts had the higher percentage of waxy seeds. The difference between these differences is  $2.4 \pm 0.7$ , which may be considered significant. No such difference between butts and tips in the percentage of waxy seeds is found when pollen from homozygous waxy plants is used on ears heterozygous for the waxy gene. In this same population the plants that furnished the heterozygous pollen for storage treatments were pollinated with homozygous waxy pollen, and the seeds from the butts and from the tips were separately classified. There were 118 of these ears, with a mean difference between butts and tips of  $0.3 \pm 0.3$  in the percentage of waxy seeds, the butts having the higher percentage.

In the comparison between the percentage of waxy seeds in the butts and in the tips of stored and fresh pollen, not all of the ears examined came from identical pollen samples, for in some cases the fresh half of the pollen sample produced ears when the stored half failed, and in other cases the reverse was true. There were, however, 17 cases where paired pollinations produced ears, one from the fresh and the other from the stored half of the pollen. Restricting the comparison of the percentage of waxy seeds in the butts and in the tips to these 17 pairs of ears, it is found that the percentage of waxy seeds in the butts is higher than that in the tips in both lots of ears and that the difference between the differences is  $1.6 \pm 0.9$  percent, the greater difference being found where the ears resulted from the use of stored pollen. This is not a significant difference.

If the differences observed in the bulk populations are due largely to reduction in pollen vitality by conditions operating during storage, it should be found in this population that the ears with fewest seeds had the greatest differences between butts and tips in the percentage of waxy seeds. This relationship would not be very close, as many factors other than pollen vitality influence the total number of seeds produced on an ear. The product-moment coefficient of correlation between the number of seeds and the magnitude of the difference in percentage of waxy seeds between butts and tips is found to be  $-0.245$ , indicating that, as the number of seeds increased, the percentage of waxy seeds in the butts and tips tended to reach equality. This coefficient, however, is not significant,  $P$  falling between 0.1 and 0.05.

In the bulk populations, it is found that in the group of 56 ears resulting from stored pollen there is a correlation of  $0.701 \pm 0.046$  between the percentage of waxy seeds in the butts and that in the tips. In the group of 31 ears resulting from the use of fresh pollen this correlation is  $0.628 \pm 0.075$ . In the group of 118 ears resulting from the use of homozygous waxy pollen on ears heterozygous for waxy, the correlation is  $-0.017 \pm 0.062$ . Clearly, the relationship between the two halves of the ear in percentage of waxy is much closer where pollen is involved than where the distribution of seeds is determined by the formation of the ovules.

Since in this material each main sample represented a single heterozygous plant, it might be argued that the plants differed with respect to the production of viable waxy pollen grains. It is more probable, however, that the viability of pollen was changed by some condition subsequent to anthesis. With stored pollen, where an

artificial difference has been interposed between the several lots of pollen the intraclass correlation is marked. It should be borne in mind that in the two series of ears resulting from heterozygous pollen essentially the same plants are involved as pollen parents. The stored pollen was identical with that of fresh pollen insofar as it was possible to insure identity. However, it is equally clear that although the pollen samples were stored in the sunlight under as nearly identical conditions as possible some condition was present that served to differentiate the separate lots.

Clearly something operates during storage that tends to make the ears pollinated by a subsample from a given lot of pollen more nearly alike than would be expected from chance alone. In other words the lots of stored pollen have become differentiated. This could be explained on the assumption that the samples differed in moisture content at the time of collection, a condition that would have no effect on the pollen used immediately but might be an important factor in its reaction to storage. Many other explanations suggest themselves, e. g., that some lots contained more pollen than others, thus providing a variable pollen screen affording the lower layers a variable protection from the sun. Variation in quantity would have little effect on pollen applied immediately. Speculation as to the cause or causes underlying the observed differences in degree of intraclass correlation are futile, but the existence of these differences is further evidence that storage (whatever the factors involved) has a differential effect on these two sorts of pollen grains.

#### DISCUSSION

Although the storage experiments conducted with pollen samples from maize plants heterozygous for the waxy gene have not resulted in demonstrating any regularity of behavior of pollen grains, there is no question that the proportions of the two sorts of grains functioning in fertilization can be altered by external agencies. It is not possible to outline at this time a treatment of pollen that will produce a given percentage of waxy seeds, but it may be stated with certainty that exposure to light will usually result in an increased effectiveness of waxy pollen grains. Were it not for the results obtained with composite pollen samples in one season, a like conclusion could be reached with respect to heat treatments in the absence of light. As the data now stand, heat treatments may either increase or decrease the effectiveness of waxy pollen. Under the circumstances, it seems more logical to assume that some factor other than the treatments given caused the deficiency in the composite samples, in which case it might be assumed that heat in the absence of light also increases the effectiveness of waxy pollen grains.

Whatever process takes place in the treated pollen grains it has been demonstrated that in some instances time can be substituted for light or heat, with similar results. The fact that storage in the dark at ordinary temperatures sometimes brings about an unequal functioning of the two sorts of grains points in the direction of an oxidation process that is speeded by illumination or high-temperatures.

Brink (3) has shown that the waxy carbohydrate has a lower diastatic activity than ordinary cornstarch, and in harmony with this finding Sprague (21) shows that pollen grains containing waxy starch

require more time for germination than do grains containing ordinary starch. If fresh waxy and horny pollen grains are applied to stigmas in quantities far in excess of the number of ovules, the result of the differential rate of germination will be a deficiency of waxy seeds. However, if small quantities of pollen were used the difference between waxy and horny pollen in the time required for germination would not be reflected in the seed classes, since competition for ovules would be largely eliminated.

If storage conditions operate merely to kill most of the pollen grains, then pollinations made with stored samples would in effect be made with greatly reduced numbers of effective grains and the two types of seed should approximate equality more nearly than when fresh pollen is used.

To account for a deficiency of waxy seeds from the use of fresh pollen and an excess from the use of stored pollen, it has been suggested (12) that the two sorts of grains were not equally mature at anthesis (a condition that might also explain the difference in time required for their germination) and that before waxy grains have reached maturity death has begun to eliminate horny grains.

Sprague's experiments offer the possibility that the conditions of storage overcome whatever factors operate to delay germination of the waxy grains and that the rate of germination of grains of this type is actually accelerated beyond that of the horny grains. This explanation may be merely substituting germination for maturity, but its greater specificity makes it attractive.

In this connection the experiments of Flint (9) on the role of light in the germination of lettuce seeds are of interest. Flint shows that light in the red end of the spectrum is essential for germination in certain samples of seed and that any sample of lettuce seed can be made to require red light for germination by a short exposure to radiation in the blue end of the spectrum.

Although it is a far cry from the germination of lettuce seeds to the differential functioning of two sorts of pollen grains of maize, it is quite possible that similar photochemical processes are involved. Flint's work shows that the wave lengths affecting germination in lettuce are close to if not identical with those that affect the development of rancidity in oils and that these regions of the spectrum have an important function in photosynthesis.

It may be that the photochemical process which differentiates between waxy and horny pollen grains is operating to oxidize fats, in which case the waxy gene must have an effect on the type or quantity of stored fats in addition to its well-recognized effect on the type of starch.

The chief drawback to this hypothesis is found in the loss of vitality of stored pollen grains. That storage conditions do kill most of the pollen grains hardly needs affirmation, but that the death rate differentiates between the two types of pollen can be inferred only from the results.

Attempts to estimate the correlation of the loss in germinating power with the time of exposure to deleterious conditions by germinating pollen samples on excised silks have not been very successful, although the tests show clearly that vitality declines. With uninjured pollen a casual inspection of silks a few minutes after pollination is enough to disclose many germinated grains, but with



treated pollen much hunting about may be required before a single germinated grain is found. Of course there is no method of distinguishing the waxy from the horny grains in a heterozygous sample without destroying their vitality. It is, therefore, not possible to measure directly any differential change in rate of germination between these two kinds of pollen grains that might result from storage.

Information on this point is very limited, for germination tests of pollen were made simply to obtain an immediate check on the lethal effect of treatments. Pollinated silks were examined and records made as to whether or not germinated grains were found. If no such grains were discovered 15 minutes after pollination, another sample was examined in half an hour, and this scrutiny was resumed at half-hour intervals up to 3 hours. No effort was made to arrive at percentages of germination, the records simply noting relative degrees of germination. These records do show, however, that some samples in which no germinated grains could be found after the first 15 minutes did in fact have a few germinated grains after 2 hours. Such observations lend little support to the hypothesis that storage treatments accelerate germination.

Although, in general, stored pollen showed less germinated grains than fresh pollen and a slower rate of germination, as measured by the elapsed time before germinated grains could be found, the method is not wholly satisfactory. In several cases good ears were produced with pollen showing no germination on excised silks, and in other cases the reverse was true.

Attempts to find a physical difference between waxy and horny pollen grains were without success. This is rather surprising, as it seems improbable that such a distinctive chemical difference would not be reflected in some measure of size. Indeed, in the seeds a difference is found in weight (waxy seeds being lighter), and since there is no difference in specific gravity waxy seeds are smaller in volume than horny seeds from the same ear (7). The difference in volume between these two sorts of seeds approximates 5 percent of the waxy seeds. Had a similar difference existed in the pollen grains it should have been disclosed in the tests with sieves and the micrometer measurements. The errors in the determinations of size of pollen grains were such that differences of the order of 3 percent could have been detected.

Although it appears impossible to separate waxy from horny pollen grains by physical means, Mangelsdorf (16, 17) has shown such separations to be possible with other differentiating genes in maize, and the process may be a general one in constant operation. With the brief time required for the germination of maize pollen (21), even slight differences in size or specific gravity (which would affect the time of their deposition on the stigmas) might have selective importance.

In the case of the waxy gene enough evidence has been presented to show that under certain adverse conditions this gene provides a survival factor for the pollen grains carrying it. There can be little doubt that under the proper conditions only waxy pollen grains would survive to effect fertilization, and probably there are many other genes that condition similar reactions. With the enormous populations of pollen grains provided by every corn plant, an oppor-

tunity is present for rigorous selection, although there is no evidence that the pollen grains surviving unfavorable environments contribute unusual vigor to the resulting zygotes.

#### SUMMARY AND CONCLUSIONS

No differences in size, weight, or specific gravity were found between waxy and horny pollen grains.

There is some evidence of a deficiency of functioning waxy pollen grains among the grains of smallest size.

There is no evidence that waxy pollen grains are less mature when shed early in the morning than when released later in the day.

Exposure of pollen grains from plants heterozygous for the waxy gene to direct sunlight for several hours increases the proportion of waxy grains that effect fertilization in those samples where this treatment is not lethal.

Similar though more erratic results are obtained when such pollen is stored in the dark for 1 or 2 hours at temperatures exceeding 40° C.

Exposure for 1 hour to artificial light of 0.1 the intensity of direct sunlight increases the proportion of waxy pollen grains that function in fertilization.

There is some indication that storage at high temperature and also in oxygen reduces the proportion of waxy pollen grains that can effect fertilization, but since this result has been obtained only with a single composite pollen sample collected from many plants it is thought that the method of compounding the pollen, rather than the storage treatment, was the causal factor.

The greatest change in the relative effectiveness of waxy pollen grains is found when pollen is exposed both to high temperatures and to light.

Storage in the dark at low temperatures (10° C.) produces no measurable effect.

Pollen samples collected from individual plants were found to react differently to the several treatments.

Pollen collected in the afternoon and applied the following morning showed no change in the relative effectiveness of waxy and horny grains.

Pollen collected in the morning and held in the dark for 24 hours at room temperature occasionally showed an increased effectiveness of the waxy grains.

When stored pollen is used the percentage of waxy seeds is higher in the butts than in the tips of the ears, indicating a relative reduction in the vitality of horny pollen grains.

A single population was obtained that showed conclusively an effect of the female parent on the behavior of pollen grains subjected to storage. The source of pollen was the same for all ears, and the ears resulting from the use of fresh pollen had similar percentages of waxy seeds, all slightly in defect of equality. In one of the groups of ears resulting from the use of stored pollen, the percentage of waxy seeds was less than that obtained with fresh pollen, whereas in the other group of ears stored pollen resulted in a higher percentage of waxy seeds than was obtained with fresh pollen.

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# WIDESPREAD OCCURRENCE AND ORIGIN OF FATUOIDS IN FULGHUM OATS<sup>1</sup>

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## INTRODUCTION

Fatuoids in oats (*Avena* L.) have been recognized for about 50 years (13, 20)<sup>2</sup> and have been of especial interest for more than 25 years because of (1) their economic importance and (2) the nature of their origin. At present there is more interest in the fatuoid than in any other aberrant in the small grains. The fatuoid, or false wild oat, usually is similar to the variety in which it occurs and is characterized by lemmas having awns and basal characters similar to the lemmas of *A. fatua* L.

The present paper is a further report on a study by the writers (3) to show the distribution of the fatuoid in the Fulghum variety and the role played by natural crossing in the occurrence of fatuoids. Fulghum was chosen because it is generally recognized as containing more fatuoids than any other agriculturally important American oat variety.

## REVIEW OF LITERATURE

The possibility of fatuoids becoming economic weeds, such as *Avena fatua*, has been mentioned by several investigators (8, 28) as well as in press dispatches. The origin of fatuoids is commonly attributed either to natural crossing (1, 29, 30, 31, 32, 33) or to a form of mutation (3, 5, 6, 8, 9, 10, 14, 15, 16, 17, 18, 19, 22, 23, 24, 25). Since 1907 the mutation theory has gained many adherents and the natural crossing but few. However, in 1929, Tschermak (31), after more than 15 years of study of the problem, asks proof from the mutationists as to the purity of their populations. That Tschermak has grounds for questioning the purity of an open-pollinated oat population is known from natural-crossing studies (11, 12, 27). In addition, Coffman and Wiebe (4) have observed examples of apparently natural crossing between *A. sativa* and *A. fatua*, and Aamodt, Johnson, and Manson (1) have conducted extensive studies of natural crossing between these two species.

The results of inheritance studies so far reported indicate that fatuoids are recessive to normal oats, usually by a single-factor difference. The writers have observed this to be true in crosses of the common, or "A type", fatuoid in Fulghum with varieties of either *Avena sativa* L. or *A. byzantina* C. Koch. The intermediate and normal forms are inseparable in some crosses.

<sup>1</sup> Received for publication Sept. 16, 1935; issued March 1936.

<sup>2</sup> Reference is made by number (italic) to Literature Cited, p. 130.

## PREVALENCE OF FATUOIDS

The cultivated red oat (*Avena byzantina*), to which Fulghum belongs, is considered by Coffman, Parker, and Quisenberry (2) to be a derivative of *A. sterilis* L., and at least two varieties belonging to that species, namely, Burt and Fulghum, contain many diverse forms, the origin of which is difficult to explain. The difficulty in obtaining true-breeding Fulghum has been reported by Stadler and Kirkpatrick (26), Parker, as reported by Farrell (7), and others. Progenies of single Fulghum panicles frequently contain fatuoids 2 or 3 years after selection, and possibly no selection of extensively grown Fulghum is free from fatuoids.

The information on the prevalence of fatuoids presented in table 1 was obtained at 28 experiment stations in 17 States of the United

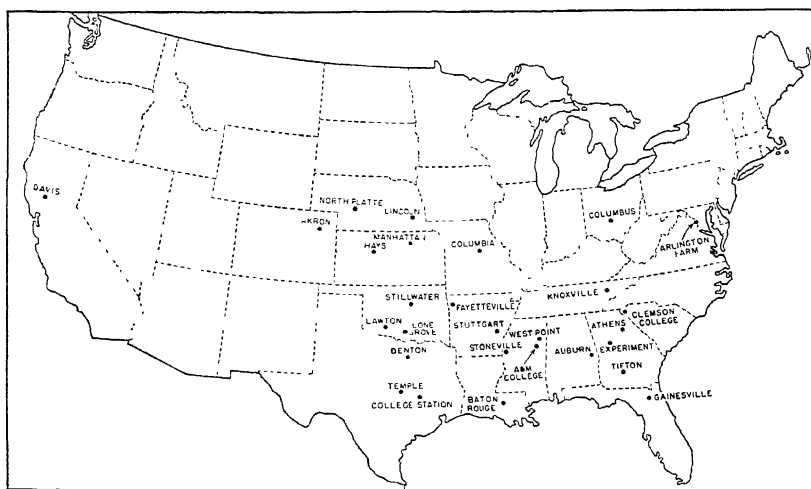


FIGURE 1.—Location of experiment stations in the United States where the presence of fatuoids in Fulghum oats has been investigated.

States (1) by questionnaires sent to experiment stations, (2) by examination of seed samples of Fulghum oats received from these stations, (3) by growing and observing plants grown from this seed at Arlington Experiment Farm, Rosslyn, Va., and (4) by observations made at the experiment stations themselves. As shown in figure 1, stations in all of the important Fulghum-producing States were included in this survey.

In 19 of the 25 samples of seed received from 18 stations, including Arlington Farm, fatuoids were found ranging from 0 to 12 per 2,000 kernels counted and averaging 0.11 percent for the 50,000 kernels examined.

When these seed samples were sown in plots in 1930, it was observed that 20 of the 25 seed samples produced some fatuoid plants. The number ranged from 0 to 9 per 2,000 individuals and averaged 0.22 percent in the 41,022 plants observed. The following year a second seeding was grown from 20 of these 25 samples. Progeny from 8 of these samples contained fatuoids, ranging from 0 to 0.67 percent per

plot. Poor germination resulted in thin stands, but, among 11,924 plants examined, 16, or 0.13 percent, were fatuoids.

TABLE 1.—*Summary of data on occurrence of fatuoids in Fulghum oats at various experiment stations during several seasons*

Item	Sta- tions repre- sented	Sam- ples sub- mitted	Reports or observa- tions on—			Indi- viduals exam- ined	Fatuoids found		
			Fatu- oids present	Fatu- oids absent	Total				
	Num- ber 17	Num- ber	Num- ber 13	Num- ber 4	Num- ber 17	Num- ber	Num- ber	Per- cent <sup>1</sup>	Per- cent <sup>2</sup>
Questionnaires.....									
Seed samples:									
Fall-sown.....	8	12				24,000	22	0.092	
Spring-sown.....	10	13				26,000	33	.127	
Total.....	18	25	19	6	25	50,000	55	.110	0-0 60
Plants grown:									
Arlington Experiment Farm, 1930: <sup>3</sup>									
Fall-sown.....	8	12				18,734	37	.198	
Spring-sown.....	10	13				22,288	52	.233	
Total.....	18	25	20	5	25	41,022	89	.217	0- 45
Arlington Experiment Farm, 1931: <sup>4</sup>			8	12	20	11,924	16	.134	0- .67
Experiment stations, 1931: <sup>5</sup>									
Fall-sown.....	14	14				12,800	42	.328	
Spring-sown.....	4	4				3,500	22	.629	
Total.....	18	18	18	0	18	16,300	64	.393	10- 90
Total fall-sown <sup>6</sup> .....						55,534	101	.182	
Total spring-sown <sup>7</sup> .....						51,788	107	.207	
Grand total <sup>8</sup> .....						119,246	224	.187	

<sup>1</sup> Average.

<sup>2</sup> Range.

<sup>3</sup> 25 samples of seed from 18 experiment stations (including Arlington) were sown at the Arlington Experiment Farm, Rosslyn, Va.

<sup>4</sup> Second seeding of 20 of the seed samples made at Arlington Experiment Farm

<sup>5</sup> Independent observations made at 18 southern experiment stations.

<sup>6</sup> Includes fall-sown seed and plants

<sup>7</sup> Includes spring-sown seed and plants.

<sup>8</sup> Includes total fall-sown, total spring-sown, and plants grown at Rosslyn, Va., in 1931.

Observations made on 18 southern experiment stations in the summer of 1931 disclosed the presence of fatuoids at all stations. The number ranged from 1 to 9 per 1,000 individuals and averaged 0.39 percent of the 16,300 plants examined.

As a result of several years' studies it was found that a few fatuoids were present in Fulghum at all 28 stations in 17 States; that they were not more prevalent in fall-sown than in spring-sown oat areas; and that the number never equalled 1 percent at any station and averaged only 0.19 percent in 119,246 individual seeds or plants. It may be concluded, therefore, that fatuoids are not now weeds in the United States, nor are they likely to become weeds under present cultural conditions.

#### ORIGIN OF FATUOIDS

##### CONSTANCY OF FULGHUM WHEN SELFED

Selfing studies were conducted to measure the importance of natural crossing on the constancy of Fulghum and fatuoids. Individual pan-



icles were enclosed in glassine bags before flowering and the progenies from such panicles were grown.

Results from selfing studies begun in 1927 on Fulghum are presented in table 2. In 1927, 97 Fulghum plants were selfed. These furnished material for later studies. The number of progenies in which no fatuoids occurred after 1, 2, 3, and 5 years of selfing, respectively, were as follows: In 1928, 300; in 1929, 220; in 1930, 216; in 1932, 88. However, of 930 Fulghum individuals grown in 1931, the progeny of 4 years of selfing, 3 were homozygous fatuoids. In 1,754 individuals produced in 5 years, 3, or 0.2 percent, were fatuoids.

The infrequent occurrence of fatuoids indicated that larger populations were desirable, and therefore Fulghum lines open-pollinated for a single season after previous bagging were grown a second year.

No fatuoids due to natural crossing would be expected in Fulghum lines open-pollinated for but 1 year, as the fatuoid is recessive to Fulghum.

TABLE 2.—Occurrence of aberrant individuals in self- and open-pollinated Fulghum oats at Arlington Experiment Farm, Rosslyn, Va., in the years 1928–32

Year examined <sup>1</sup>	Normal (previously selfed) <sup>2</sup>				Fatuoid progeny plants						
	Parent plants	Progeny plants				Previously selfed			Previously open-pollinated		
		Total	Fatuoids			Total	Aberrant		Total	Aberrant	
	Number	Number	Number	Per cent	Number	Number	Per cent		Number	Number	Per cent
1928.....	20	300	0	0 0	340	0	0		1,402	16	1 14
1929.....	9	220	0	.0	105	0	0		132	2	1 51
1930.....	10	216	0	0	422	0	0		162	5	3 09
1931.....	98	930	3	.3	168	0	0		676	46	6 80
1932.....	8	88	0	0	21	0	0		587	275	46 85
Total.....	145	1,754	3	.2	1,056	0	0		2,959	344	11.6

<sup>1</sup> Aberrances observed are the result of mutations or natural crosses that occurred the previous year.

<sup>2</sup> Less than 0.5 percent of aberrant plants were observed in the open-pollinated Fulghum.

In 1930 there were grown 1,198 progeny individuals, the parents of which had been bagged for two generations, i. e., 1927 and 1928, but had been open-pollinated in 1929. In 1931 there were grown 9,856 progeny individuals, the parents of which had been bagged in 1927, 1928, and 1929 but had been open-pollinated in 1930. Six fatuoids were observed in the 1,198 individuals produced in 1930, and 20 in 1931, a total of 26, or 0.2 percent, in the 11,054 plants. The 1,198 progeny plants produced in 1930 were from head-to-row seedlings and 6 of the rows contained fatuoids. In 1931 a progeny row was grown of each individual in these 6 rows and the resulting test proved that the 6 strains were similar in breeding behavior to  $F_3$  populations of a cross of normal  $\times$  fatuoid which differed by a single factor. As natural crossing was excluded by selfing in 1928, it is apparent that chromosomal irregularities occurred that year, giving in 1929 the intermediate type that segregated in 1930.

All six mutations observed in 1930 apparently were to the intermediate form, although Marquand (21) and Huskins (17) have reported the observation of mutation direct to the homozygous form. This latter is possible when aberration occurs in both the male and the female sex cells that form the zygote.

However, if fatuoids arise only by mutation and 0.2 percent of these aberrances occur in a population, it would theoretically require 15,625 individuals for the mutation to occur directly to the homozygous form, whereas mutation to the heterozygous form would occur once in each 125 individuals.

#### CONSTANCY OF THE FATUOID

It has been reported that the Fulghum fatuoid does not always breed true (7, 28). Plants of fatuoids were selfed to test their behavior under controlled pollination. Progenies were grown from the selfed fatuoid panicles as follows: 340 in 1928, 105 in 1929, 422 in 1930, 168 in 1931, and 21 in 1932. The progeny of all these 1,056 bagged fatuoids bred true.

In addition to the selfing studies, progenies from fatuoids of the same lines were grown open-pollinated. Adjacent to the fatuoid lines were rows of Fulghum and Black Mesdag. The  $F_1$  of a natural cross of these varieties on the fatuoid is easily recognized by the dominance of their characters. Intermediate or  $F_1$  plants were observed as follows: In 1928, 16 of 1,402; in 1929, 2 of 132; in 1930, 5 of 162; in 1931, 46 of 676; and in 1932, 275 of 587. Of 2,959 individuals, 344, or an average of 11.6 percent, apparently were crosses. In 1932, more than 46.8 percent of the progeny of open-pollinated fatuoids apparently were crosses. Therefore, it may be stated that the common or A type fatuoid in Fulghum shows a varying tendency to be cross-pollinated unless foreign pollen is excluded.

The high frequency of cross-pollination in Fulghum fatuoids is striking when compared with the low frequency in normal Fulghum. Less than 1 percent of cross-pollination was observed in the normal Fulghum in these experiments as compared with an average of 11.6 percent and a maximum of nearly 47 percent in the fatuoids in a single season. Of course, factors of climate and soil may influence pollination.

#### VIGOR OF THE FATUOID

Several investigators have stated that there was little difference in vigor between the normal and the A type Fulghum fatuoid. The data in tables 3 and 4 indicate that plants of fatuoid Fulghum are shorter than those of normal Fulghum and bear more than twice as many infertile florets. The average height of the fatuoids was  $38.8 \pm 0.06$  inches; of the intermediate fatuoids,  $40.6 \pm 0.06$  inches; and of the apparently normal plants,  $40.7 \pm 0.09$  inches. It is not always possible to differentiate the intermediate fatuoid from the normal plant.

TABLE 3.—*Relative height of normal, intermediate, and fatuoid Fulghum plants in segregating populations*

Type of plant	Plants	Height of plant	Tillers per plant
	<i>Number</i>	<i>Inches</i>	<i>Number</i>
Normal.....	136	$40.7 \pm 0.085$	$3.5 \pm 0.069$
Intermediate <sup>1</sup> .....	249	$40.6 \pm .062$	$3.9 \pm .061$
Fatuoid.....	107	$38.8 \pm .064$	$4.0 \pm .124$

<sup>1</sup> It is often difficult to separate intermediate and normal forms.

TABLE 4.—Relative fertility of florets on open-pollinated, primary, secondary, and self-pollinated panicles of normal and fatuoid Fulghum oats, 1927-31

Crop year	Florets on normal panicles of indicated type						Florets on fatuoid panicles of indicated type					
	Primary		Secondary		Selfed		Primary		Secondary		Selfed <sup>1</sup>	
	Total	Sterile	Total	Sterile	Total	Sterile	Total	Sterile	Total	Sterile	Total	Sterile
	Number	Percent	Number	Percent	Number	Percent	Number	Percent	Number	Percent	Number	Percent
1927.....	2,329	5.8	1,236	16.7	751	30.2	1,345	11.5	1,330	35.7	2,236	43.9
1928.....	482	19.7	510	28.4	338	29.6	162	51.2	493	75.3	879	49.1
1929.....	503	7.8	143	18.9	371	33.2	302	34.1	167	58.1	621	74.4
1930.....	340	9.4	220	30.5	805	34.8	103	41.7	117	29.1	978	77.2
1931.....	241	7.0	145	49.7	231	44.6	149	25.5	407	52.1	353	83.6
Total or weighted average.....	3,895	8.2	2,254	23.0	2,496	33.4	2,061	20.5	2,514	47.3	5,067	57.7

<sup>1</sup> Panicles of primary type

In the fatuoid and the apparently normal Fulghum plants, 20.5 and 8.2 percent, respectively, of the florets of primary panicles were sterile. In both types of plants more than twice as many florets were sterile in secondary panicles as were sterile in primary panicles. Also, more than twice as many florets were sterile in the primary and secondary panicles of the fatuoid plants as were sterile in these respective panicle types in the normal Fulghum. This high percentage of sterility in fatuoids probably is one reason why Fulghum fatuoids cross-fertilize so frequently and often do not breed true when open-pollinated.

Data obtained on Black Mesdag, grown to compare its fertility with that of Fulghum and the fatuoids, indicated that bagging did not seriously impair fertilization in this *Avena sativa* variety. Data were obtained in each of 4 years. Of a total of 1,563 florets on primary and 647 on secondary panicles, an average of 21.6 and 41.7 percent of the florets were sterile on the primary and secondary panicles, respectively. The range in sterility was from 2.7 percent in 1931 to 34.3 percent in 1930 in the primary and from 13.6 in 1929 to 77.0 percent in 1930 in the secondary panicles. Panicles were bagged in 1928 and 1930. An average of 19 percent of 667 florets were sterile, i. e., 14.3 percent in 1928 and 20.8 percent in 1930.

The fact that fatuoids appear to be slightly less vigorous than normal Fulghum plants probably explains the observed decrease in the number of fatuoids in fall-sown Fulghum and Kanota varietal plots grown at Arlington Experiment Farm. At probably no experiment station included in this study are Fulghum and Kanota grown from fall seeding under conditions so apt to result in winter-killing as at Arlington Farm. The decrease in the number of fatuoids to the one-fortieth-acre plot is indicated by the data in table 5. No data are available on the number of plants per plot, but the trend seems to be very pronounced.

TABLE 5.—*Occurrence of fatuoids in Kanota and Fulghum oats grown at Arlington Experiment Farm, Rosslyn, Va., from seed received from Kansas*

Variety	Date introduced	Fatuoids per 1/40-acre plot in—				
		1923	1924	1927	1928	1929
Kanota.....	1922	Number 20 0	Number 0 8	Number 1 0	Number 0 3	Number 1 7
Fulghum.....	1925	.....	14 0	15 0	2 3	1 7

## SUMMARY AND CONCLUSIONS

Interest in fatuoids apparently has existed for 50 years and controversy as to their origin for more than 25 years. Of the two explanations offered for their origin, that of mutation is more widely accepted than that of natural crossing between *Avena fatua* and cultivated oats.

Fatuoids were present in Fulghum oats at 28 experiment stations in 17 States of the United States and were not more numerous in the fall-sown than in the spring-sown oat areas. They were nowhere sufficiently numerous to be an economic factor as weeds. They constituted less than 1 percent of any population of Fulghum oats examined. Fatuoids are detrimental chiefly because their presence may discredit otherwise promising oat strains or seed samples.

Approximately 0.2 percent of fatuoids appeared spontaneously in lines of normal Fulghum that had been self-pollinated for four generations. In all cases the mutation apparently occurred to the intermediate type, resulting, in the following generation, in the production of fatuoid and normal plants in a ratio of 1 : 3. When selfed, the Fulghum fatuoid bred true.

Theoretically, the mutation occurred to the intermediate form once in 125 individuals. Mutation to the homozygous form would be expected to occur once in 15,625 individuals.

Data obtained from 5 years' study show that when the fatuoid was open-pollinated it was cross-fertilized on an average 11.6 percent and that a maximum of nearly 47 percent of cross-fertilization occurred in a single season. Under similar conditions, normal Fulghum, recognized as being relatively subject to natural crossing, contained less than 0.5 percent of crosses in any season.

The data indicate that the ordinary or A type fatuoid in Fulghum is less vigorous and more readily cross-fertilized than is the normal plant. This may partly account for the susceptibility of the fatuoid to cross-pollination and may be considered evidence that the persistence of fatuoids in normal oats is due in part to natural crossing.

Covering the panicles with glassine paper bags to prevent cross-pollination reduced fertility in all cases. The percentage of decrease was least in Black Mesdag and greatest in the fatuoid.

Fatuoids of the common or A type are slightly less vigorous than are normal Fulghum plants. This may explain the apparent reduction in number of fatuoids per plot of Fulghum grown on Arlington Experiment Farm from fall seeding.

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# THE RELATIONSHIP OF THE PHOSPHATE CONCENTRATION OF SOLUTION CULTURES TO THE TYPE AND SIZE OF ROOT SYSTEMS AND THE TIME OF MATURITY OF CERTAIN PLANTS<sup>1</sup>

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## INTRODUCTION

In an extended study of the nutrition of plants, a number of observations and studies may be made, definite results can be obtained, and conclusions drawn on some of them long before the work is concluded. In such a study on phosphorus nutrition three phases have already given rather definite results and it is deemed well to report them before the project is concluded. They are as follows: (1) The interrelationship between the size and type of root system, the phosphate concentration, and the amount of growth; (2) the size of root system as affected by the concentration of phosphate in the nutrient medium; and (3) the effect of different phosphate concentrations on the time of maturity.

For the purpose of these investigations plants of various kinds were grown in a series of tanks containing solutions with phosphate concentrations maintained at various constant levels. These cultures demonstrated that, all other conditions being the same, the minimum concentration for satisfactory growth varied with the kind of plant. The reason for such a difference may be either that one kind of plant may require a greater phosphate concentration in its nutrient media because of a difference in the amount necessary for physiological activities, or there may be a difference in the rate at which the root systems absorb the necessary ion, or both. Observations indicated that the type and extent of the root system might be an important factor. Experiments, therefore, were carried out to determine whether a relatively high concentration of phosphate was not often necessary, on account of the small extent of surface exposed, to supply the ion at a rate sufficiently rapid for satisfactory growth.

Observations on the root systems of the plants grown in these solutions indicated that higher phosphate concentrations did not stimulate root development, that is, did not cause a greater percentage increase for the roots than for the tops. Most studies in the past on the effect of phosphate on root systems took into consideration only increases in root weight and did not compare these increases with those of the tops. If the relative increase of the tops is as great as, or greater than that of the roots, a considerable part of the increase in the size of the roots of plants grown at higher phosphate concentrations may be due to the greater amount of food synthesized. Studies were therefore made in which the root-top ratio (weight) instead of absolute weights was taken as the criterion to determine whether or not phosphate actually stimulated root development.

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Observations that a number of plants in solutions of low phosphate concentrations matured earlier than those in solutions of higher phosphate concentration led to a study of the effect of phosphate concentration on the time of maturity. The results of the study are reported herein.

#### PROCEDURE

The following plants were used in the experiments: Peas, buckwheat, corn, tomatoes, wheat, and cotton.

All plants were grown in solutions in 1,000-liter galvanized-iron tanks. These tanks were 4 feet in diameter and were coated on the inside with asphalt paint. The original composition of the solutions was the same for all constituents excepting monobasic potassium phosphate. The initial solution was made up as follows:

Salts:	Grams per 1,000 liters of tap water
$\text{KNO}_3$ .....	400
$\text{CaSO}_4 \cdot 2\text{H}_2\text{O}$ .....	250
$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ .....	150
$\text{MnSO}_4 \cdot 2\text{H}_2\text{O}$ .....	3. 5
$\text{H}_3\text{BO}_3$ .....	3. 5

Phosphate determinations on samples filtered through hardened filter paper were made at least once daily, and  $\text{PO}_4$  as  $\text{KH}_2\text{PO}_4$  was added to maintain the desired concentrations, varying in these experiments from 0 to 12.8 p. p. m. A continuous current of air was used to agitate the solutions so that the  $\text{PO}_4$  concentration would be uniform throughout the container. Twenty-five grams of heptahydrate ferrous sulphate ( $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ ) were added (usually once a week) throughout the experiments. It was sometimes necessary during the early stages of growth to add the iron a little more frequently to maintain a good green color of the plants. The current of air was discontinued after the addition of the iron for a period of about 20 hours to prevent the rapid oxidation of ferrous to ferric iron. The iron precipitated the  $\text{PO}_4$  almost completely so that during a period of 24 hours there was practically no  $\text{PO}_4$  in solution. The current of air was started a few hours before the  $\text{PO}_4$  was again added to oxidize the remaining ferrous iron to the ferric form so that the difference in the solubility of ferrous and ferric phosphate would not be a factor with which to reckon when restoring the  $\text{PO}_4$  concentration. Since much of the iron was precipitated as the oxide and the solution tended to become acid, 25 grams of calcium carbonate were added when aeration was resumed. The addition of the calcium carbonate also helped in the ease with which the  $\text{PO}_4$  concentration was reestablished by aiding in the precipitation of the iron. There was always undissolved calcium carbonate at the bottom of the tanks.

An excess of all nutrients excepting  $\text{PO}_4$  was maintained. In most cases the plants were moved in rotation to the various tanks. This was done weekly just before the  $\text{PO}_4$  concentration was restored. In other cases nutrient salts were added to the cultures that had grown sufficiently large to cause considerable decrease in the concentration of these salts.

The plants were supported by corks (one plant per cork) which fitted in holes in the tops of the containers.

## EXPERIMENTAL RESULTS AND DISCUSSION

## THE INTERRELATION BETWEEN THE SIZE AND TYPE OF ROOT SYSTEM, THE PHOSPHATE CONCENTRATION, AND THE AMOUNT OF GROWTH

Two types of experiments were carried out in the study of the relationship of the size and type of root system to the minimum  $\text{PO}_4$  concentration necessary for satisfactory growth. In both types different kinds of plants were grown in the same containers in which the  $\text{PO}_4$  concentrations of the solutions were maintained at various levels. In the first type a study was made of the relative size of the surface of the roots as compared with the growth of the plants. In the second type the  $\text{PO}_4$  concentration of the expressed juice of the stems between the roots and the cotyledons was determined to ascertain whether it, and therefore the rate of supply of phosphorus to the plant, was proportional to the surface of the roots exposed to the culture solution.

Eight plants each of wheat, peas, buckwheat, corn, tomatoes, and cotton were transferred to each of four containers. The phosphate concentration in two of the tanks was maintained at 0.1 p. p. m. and in the other two at 0.8 p. p. m. These concentrations were chosen because previous experiments had shown that none of these plants made maximum growth in solutions in which the phosphate concentration was maintained at 0.1 p. p. m. but showed great differences at this concentration, and because all plants made good growth at 0.8 p. p. m. although some of them did not make maximum growth. The seeds of all plants were germinated at the same time, and all seedlings excepting the tomatoes were transferred to the culture solutions on the same day. The tomato plants were too small at that time and were therefore not set out until 3 days later.

Since there is no accurate way of measuring the surface of roots, all comparisons were a matter of judgment and photographs are the only evidence presented.

Photographs were taken after the plants had been growing in solutions 23 days. In order to show their structure, the plants were placed on a tray under water while they were being photographed. The roots appear dark in the photographs because of the ferric oxide deposited on them. The ferric oxide precipitate greatly increased the visibility of the root hairs; and where they are numerous, they appear on the photographs as dark patches with hazy outlines. The roots of plants grown in solutions of low  $\text{PO}_4$  concentration (0.1 p. p. m.) are shown in figure 1. Figures 2, 3, and 4, show the comparative size of plants grown in solutions with  $\text{PO}_4$  concentrations of 0.1 and 0.8 p. p. m.

The root-top ratio (apparent surface of the roots as compared with the size of the tops) arranged in descending order for the plants grown in the solutions having a  $\text{PO}_4$  concentration of 0.1 p. p. m. is as follows: Wheat, buckwheat, peas, tomatoes, corn, and cotton. For a concentration of 0.8 p. p. m., the order was the same, with the exception of the tomato plants which grew the least. Root hairs on the wheat and buckwheat were by far the most numerous, those on tomatoes came next, thus making the order of size of root surface of these plants as compared with that of the peas rather doubtful since the peas had very few root hairs. No root hairs were found on the corn and cotton

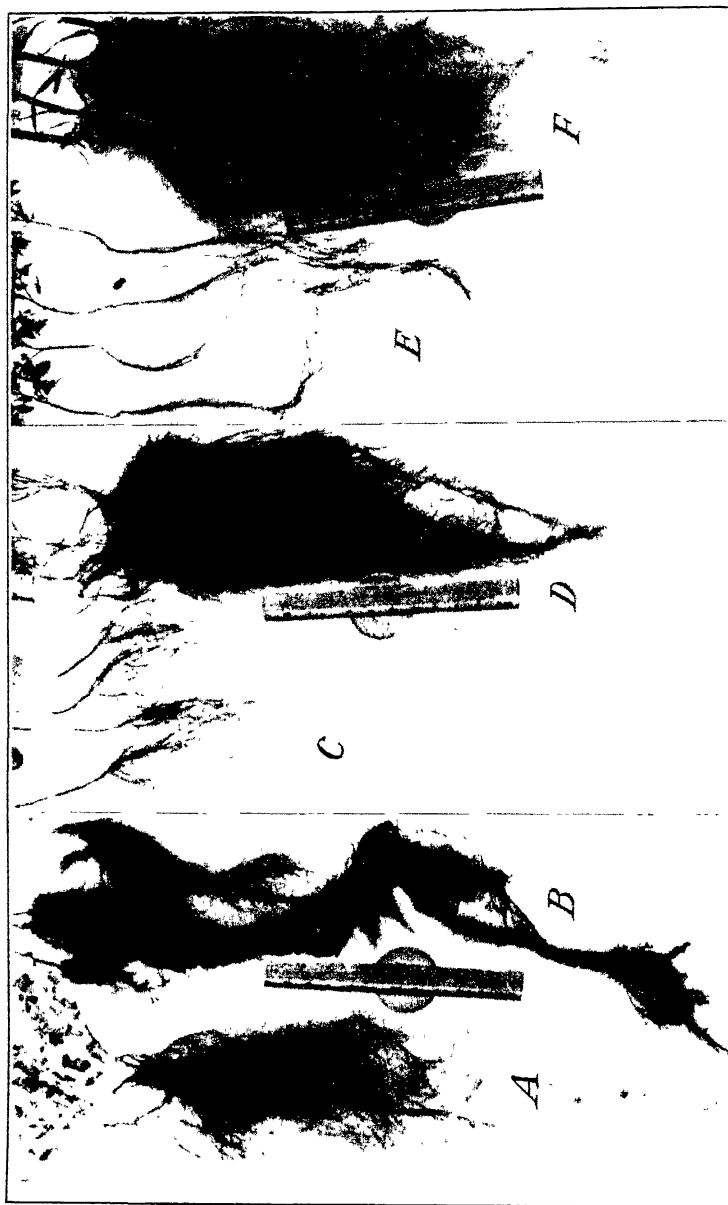


FIGURE 1.—Roots of plants grown in solutions with a  $P_2O_5$  concentration of 0.1 p. p. m. : A, Peas; B, buckwheat; C, cotton; D, wheat; E, tomatoes; F, corn

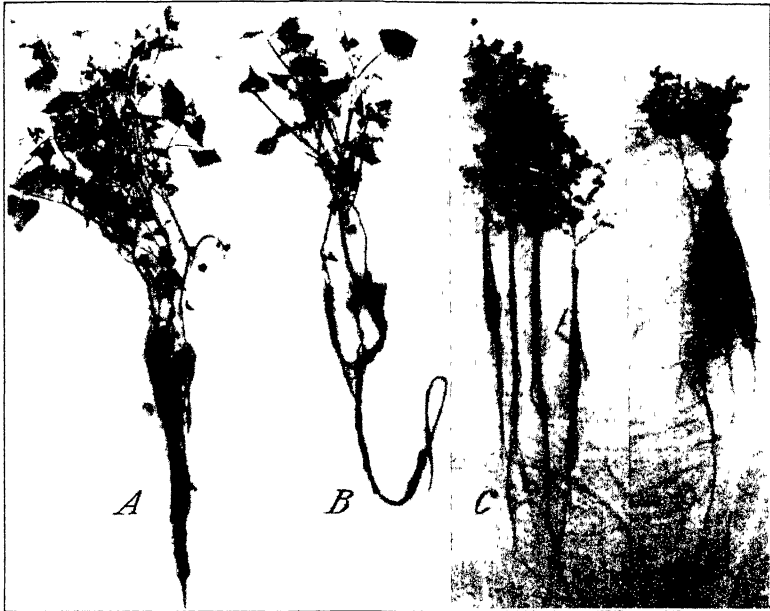


FIGURE 2.—Buckwheat (*A* and *B*) and pea plants (*C* and *D*) grown in solutions with  $\text{PO}_4$  concentrations of 0.8 (*A* and *C*) and 0.1 p. p. m. (*B* and *D*).



FIGURE 3.—Corn (*A* and *B*) and wheat plants (*C* and *D*) grown in solutions with  $\text{PO}_4$  concentrations of 0.8 (*A* and *C*) and 0.1 p. p. m. (*B* and *D*).

One group of four tomato plants became shaded by the corn during the latter part of the experiment. These plants then began to make rapid growth and when harvested were much larger than the other tomato plants in the same container and those in the other container of the same phosphate concentration.

The difference in growth of different kinds of plants grown in the same kind of solution with varying  $\text{PO}_4$  concentrations is shown in figure 5 and in table 5. The plants shown in figure 5 were transferred to the experimental solution in the cotyledon stage at the same time. A  $\text{PO}_4$  concentration of 0.2 p. p. m. was maintained.

In the second type of experiments, cotton, buckwheat, and tomato plants were used in the studies of the relative rate at which the roots

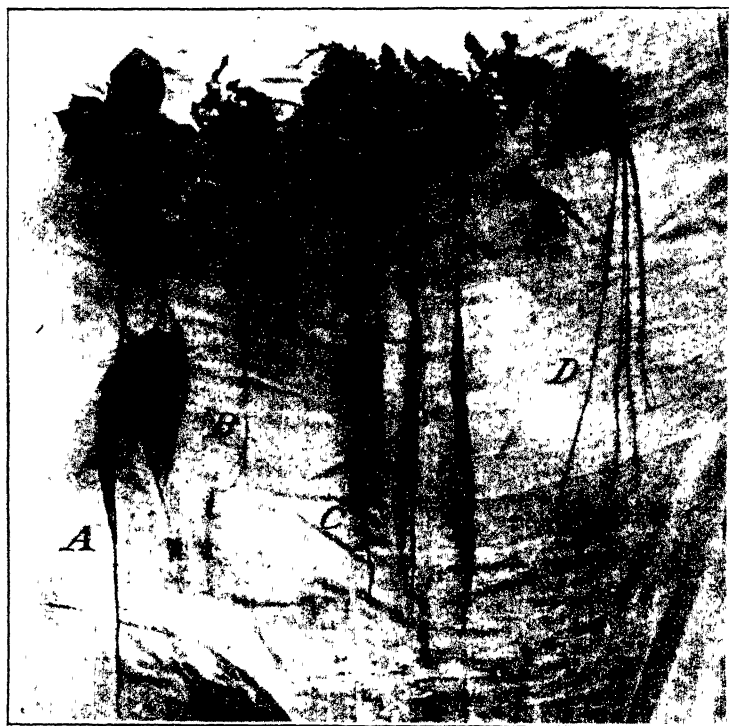


FIGURE 4.—Cotton (A and B) and tomato plants (C and D) grown in solutions with  $\text{PO}_4$  concentrations of 0.1 (B and D) and 0.8 p. p. m. (A and C).

of different plants supply  $\text{PO}_4$ , because cotton requires a relatively high  $\text{PO}_4$  concentration for good growth, whereas the other two plants grow well at much lower concentrations, and because these plants could be divided into comparable parts for analyses. In the first experiment two crops of buckwheat were grown in the same containers with cotton plants on which a study was being made to determine the minimum  $\text{PO}_4$  concentration necessary for maximum growth. Earlier experiments indicated that good growth of cotton could not be obtained at concentrations below 0.8 p. p. m. This was therefore the lowest concentration used in this experiment. Solutions of two

other  $\text{PO}_4$  concentrations, 3.2 and 12.8 p. p. m., were included. After the cotton had been growing in the containers for 28 days, the first crop of buckwheat was set out (in the cotyledon stage) and allowed to grow for 10 days when it was harvested and a second lot (also in the cotyledon stage) was placed in the solutions for the same length of time. At each buckwheat harvest some of the cotton plants were also harvested. The stems of both kinds of plants between the roots and

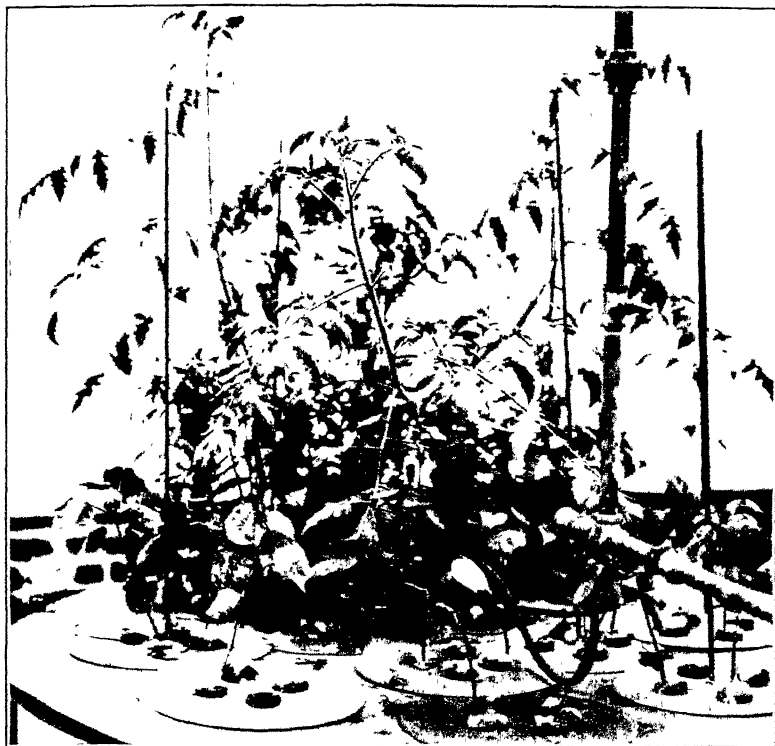


FIGURE 5.—Three tomato plants and cotton plants of the same age grown in solutions with a  $\text{PO}_4$  concentration of 0.2 p. p. m.

cotyledons were frozen for phosphorus determinations of the expressed juice, and the young leaves were dried and analyzed for phosphorus.

In a second experiment  $\text{PO}_4$  concentrations were maintained at 0.2, 0.4, 0.8, and 3.2 p. p. m. These concentrations included those at which very little growth of cotton was made and above which greater growth was not obtained. A crop of buckwheat was grown with the cotton as in the preceding experiment and when harvested tomato plants were substituted. The tomato plants were allowed to grow for 25 days when they were harvested with the remaining cotton plants. At this time there were not enough cotton plants left in solution of 0.2 p. p. m. for analyses and the tomato plants growing in solutions of higher  $\text{PO}_4$  concentrations were so shaded by the rapidly growing cotton plants that they were discarded. Analyses were made as in the preceding experiment.

In a third experiment cotton and tomato plants were germinated and transferred to the containers at the same time. Phosphate concentrations of 0.2 and 3.2 p. p. m. were maintained. Some of both kinds of plants were harvested for analyses at the end of 21, 29, and 46 days.

There was considerable difference in the amount of growth made by the tomato plants at low  $\text{PO}_4$  concentrations in the last two experiments. The weather was cloudy most of the time during the growth of the plants in the second experiment, and the tomato plants in the solutions with a low  $\text{PO}_4$  concentration appeared to make as rapid growth as those in solutions of higher  $\text{PO}_4$  concentrations. Clear weather prevailed during the first few weeks of growth of the plants of the third experiment; the tomato plants at low  $\text{PO}_4$  concentrations made poor growth and had the deep purple color that develops when phosphorus is deficient. Cloudy weather began 12 days after the second harvest. The tomato plants in solutions with a phosphate concentration of 0.2 p. p. m. immediately began to make rapid growth, water of guttation appeared the morning after each cloudy day, and the new growth was not purple.

The results of these experiments are shown in table 1. With one exception the amount of phosphorus in the expressed juice of the buckwheat stems was considerably greater than that of the cotton stems. In this exceptional case the buckwheat for the last days was shaded by the rapidly growing cotton. Where the tomato plants were making good growth, in solutions of both high and low  $\text{PO}_4$  concentration, the results were similar to those of the buckwheat but those tomato plants that were making poor growth at low  $\text{PO}_4$  concentrations had less  $\text{PO}_4$  in the expressed juice of the stems than did the cotton. Where the amount of phosphorus in the expressed juice of the stems was less for the tomato plants than for the cotton, the plants had been transferred to the culture solutions and had grown in sunny weather. Plants of the same group which were harvested after 46 days and which were harvested after a number of cloudy days contained more phosphorus than did the cotton harvested at the same time.

There appears to be some factor which, under conditions of bright sunlight and low  $\text{PO}_4$  concentration, decreases phosphate absorption for the tomato plant. Where the light intensity was sufficiently low the amount of growth of the tomato plant was that which would be expected for a plant with such a root system at low  $\text{PO}_4$  concentrations.

Further evidence of the difference in the  $\text{PO}_4$  concentration necessary for the good growth of tomato plants under different light conditions was obtained in another experiment. One group of plants grown for the first few weeks in cloudy weather in a solution with a  $\text{PO}_4$  concentration of 0.1 p. p. m. made better growth than did plants grown during sunny weather in solutions with a  $\text{PO}_4$  concentration of 0.2 p. p. m. Varying weather conditions appeared to cause different rates of growth for the plants growing in solutions with a  $\text{PO}_4$  concentration of 0.1 p. p. m. After the first few weeks of cloudy weather there were about 2 weeks in which sunny weather predominated. The tomato plants appeared to stop growing, and a purple coloration began to appear. After a day or two of cloudy weather, water of guttation again appeared and growth was resumed.

TABLE 1.—*Phosphorus in the expressed juice of stems and in young leaves of cotton, buckwheat, and tomato plants grown in cultures of various phosphate concentrations*

PO <sub>4</sub> concentration in parts per million and experiment no. <sup>1</sup>	Phosphorus in expressed juice of stems			Phosphorus in young leaves (dry weight)					
				Total			Soluble		
	Cotton	Buckwheat	Tomato	Cotton	Buckwheat	Tomato	Cotton	Buckwheat	Tomato
	<i>Percent</i>	<i>Percent</i>	<i>Percent</i>	<i>Percent</i>	<i>Percent</i>	<i>Percent</i>	<i>Percent</i>	<i>Percent</i>	<i>Percent</i>
0.2									
3.....	0.0035	0.019		0.40	0.80		0.15	0.58	
5.....	0.033		0.0021	44		0.42	0.05		0.057
5'.....	0.046		0.025	45		44			18
5''.....	.0030		.0042	25		65	14		22
0.4									
3.....	.0055	.029		58	.87		39	.57	
4.....	.0065		.0183	66		.16	40		.89
0.8									
1.....	.0090	.020		55	.76		35	.53	
2.....	.0090	.030		76	1.10		52	.86	
3.....	.0089	.024		72	1.00		48	.57	
3.2									
1.....	.0095	.034		68	.86		46	.65	
2.....	.020	.040		1.18	.88		.84	.61	
3.....	.057	.028		.99	.87		.52	.52	
5.....	.0072		.017	.68		1.09	.33		.82
5'.....	.0073		.021	.70		1.05	.41		.90
5''.....	.011		.049	.93		1.12	.27		.83
12.8									
1.....	.012	.36		1.00	.90		.68		.67
2.....	.028	.57		.99	.88		.80		.58

<sup>1</sup> 1 and 2, first experiment of test, cotton plants set out at the same time but different lots of buckwheat 3 and 4, second experiment, cotton set out at the same time, first buckwheat was set out with cotton 3, and later replaced by tomatoes 4, 5, 5', and 5'', third experiment; cotton and tomato plants all set out at the same time but harvested at 3 different times

A number of investigators have shown that plants differ greatly in their capacity to use the phosphorus of the soil. Hartwell (3, p. 7)<sup>2</sup> found that carrots secured their entire needs under conditions where turnips were practically unable to grow without phosphatic application; millet and tomatoes ranked next to carrots; and beets and rape next to turnips. Cotton requires phosphorus fertilization whereas oats will make good growth without it. The soil is so complex a system that very little has been learned from soil studies to explain these differences. With solution cultures many of the difficulties are removed, and the problem may be reduced to one of the PO<sub>4</sub> concentration necessary for the normal growth of the plant under certain conditions.

Both water culture and field experiments have shown that wheat and buckwheat are able to grow with lower PO<sub>4</sub> concentration than some other plants. Both of these plants produce an enormous number of root hairs and, therefore, have a great root surface. If judged by dry weight, buckwheat would have a very small root system, but its fine roots and numerous root hairs with little weight present a very large surface for absorption. In solution cultures tomato plants vary greatly in the number of root hairs produced, but in all experiments here have produced very large root systems with many fine roots. The minimum PO<sub>4</sub> requirement for tomato plants appears to vary markedly with the amount of light; that is, the greater the light intensity the higher the PO<sub>4</sub> concentration necessary.

<sup>2</sup> Reference is made by number (italic) to Literature Cited, p. 147



Cotton had the poorest root system of the plants studied. There were no root hairs or very fine roots on plants grown in the culture solutions. Its growth at low  $\text{PO}_4$  concentrations was also much the poorest, with the exception of tomatoes grown in bright light. In cloudy weather, at 0.2 p. p. m., it made very poor growth, while tomato plants in the same containers were making very rapid growth.

The requirements of greater amounts of phosphate for some plants than for others under field conditions can be explained only in part by solution-culture experiments. Snow (5) found that corn produced no root hairs in solution cultures but that they were numerous under normal soil conditions. This may explain the findings of Tidmore (6, 7) that certain soils producing good corn without  $\text{PO}_4$  fertilization had in their displaced solutions  $\text{PO}_4$  concentrations of only 0.03 to 0.05 p. p. m., while solution cultures with a  $\text{PO}_4$  concentration of 0.05 p. p. m. produced very poor plants. The growth of corn obtained in solutions with a  $\text{PO}_4$  concentration of 0.1 p. p. m. might be expected for such a root system. According to weight, corn in solution cultures would be considered to have a large root system, but the roots for the most part are coarse and therefore the relative surface exposed is small. Another factor probably important, especially in neutral and alkaline soils, is the relative amount of carbon dioxide ( $\text{CO}_2$ ) given off by different plants. The carbon dioxide under these conditions may serve two functions in phosphorus nutrition, that of making more soluble the insoluble phosphates, and of producing in the immediate vicinity of the root a reaction more favorable for the absorption of anions. Newton (4) found that peas absorbed more calcium than did barley when grown in soil. He found that the roots of peas produced more carbon dioxide than did those of barley and explained the greater absorption of calcium by the peas on the assumption that the solubility of the calcium was increased by the carbon dioxide, thus making more calcium available for the peas than for the barley. Breazeale and McGeorge (1) say that carbon dioxide is the most important single factor in the fertility of alkaline soils. In many cases, because of the effect of carbon dioxide, the displaced soil solution may not give a true picture of what is available for the root. It is probably impossible, moreover, to get an adequate picture of conditions where root hairs are in contact with the colloidal fraction of the soil.

THE SIZE OF THE ROOT SYSTEM AS AFFECTED BY THE CONCENTRATION OF  
PHOSPHORUS IN THE CULTURE SOLUTIONS

In this study of the effect of  $\text{PO}_4$  concentration on the size of the root system, particular attention was paid to the weight of the roots as compared with that of the tops, since it seems valid to say that phosphate fertilization stimulates root development only in case the relative increase in the weight of the roots is greater than that of the tops.

Plants were grown under two sets of conditions. In one experiment tomato, pea, cotton, wheat, and corn plants were grown in solutions of which the  $\text{PO}_4$  concentration was maintained at various low levels. In the other, tomato, wheat, corn, and cotton plants were first grown for several weeks with an adequate supply of  $\text{PO}_4$ ; some of the plants of each kind were transferred to phosphate-free solutions, while the rest were allowed to continue their development in solutions with a  $\text{PO}_4$  concentration sufficiently high for good growth.

TABLE 2.—*Root-top ratio, by weight, of plants of four kinds when grown in culture solutions of various phosphate concentrations*

Kind of plant and experiment no. <sup>1</sup>	Root-top ratio when grown at PO <sub>4</sub> concentration of—						Comments
	0 1 p p m	0 2 p p m	0 4 p p m	0 8 p p m	3 2 p p m	12 8 p p m	
Tomato							
1.....		0 50		0 33	0 28		Young plants
2.....		47			39		Do
3.....		2 37	2 0 15				Do
4.....	2 55				24		Do
5.....		54			29		Do
Pea							
1.....		06		04	04		Mature plants.
Cotton							
1.....				13	05	0 05	Do.
2.....		2 92	2 77	2 49	2 32		Young plants.
3.....			2 44	2 28	2 23		Rapidly growing plants, same as above but 1 month older
4.....		2 1 35			2 48		Between 5 and 6 weeks old
5.....		.19	.08	.08	12		Plants at 0.2, 0.4, and 0.8 p. p. m. had finished growing, plants at 3.2 p. p. m. were same age but still growing.
6.....		2 82			2 41		Young plants.
Wheat							
1.....		04		.10	.07		Plants all the same age, but those at 0.2 p. p. m. had stopped growing and produced ripe seed, those at 0.8 and 3.2 p. p. m. still making vegetative growth.
Corn							
1.....	.78	.55			19		Young plants

<sup>1</sup> Nos. 1, 2, etc., correspond to those numbers of table 3 and refer to the same plants

<sup>2</sup> Green weights, all of the others, dry weights.

TABLE 3.—*Weights of roots and tops of plants of four kinds when grown in culture solutions of various phosphate concentrations*

Kind of plant and experiment no. <sup>1</sup>	Weight of roots and tops when grown at PO <sub>4</sub> concentration of—											
	0.1 p. p. m.		0 2 p. p. m.		0 4 p. p. m.		0 8 p. p. m.		3 2 p. p. m.		12 8 p. p. m.	
	Roots	Tops	Roots	Tops	Roots	Tops	Roots	Tops	Roots	Tops	Roots	Tops
Tomato	<i>G</i>	<i>G</i>	<i>G</i>	<i>G</i>	<i>G</i>	<i>G</i>	<i>G</i>	<i>G</i>	<i>G</i>	<i>G</i>	<i>G</i>	<i>G</i>
1.....			4 9	9 9			9 8	29.9	10.7	38.0		
2.....			2 2 6	2 5 5					11.8	30.5		
3.....			2 1 8	2 4 9	2 1 5	2 9 7						
4.....	2 2 8	2 1 1							2 25 1	2 105 0		
5.....			2 26.5	2 48.9					2 143.5	2 490.5		
Pea: 1.....			.6	10 1			1.8	40 1	1.8	47.6		
Cotton												
1.....							16.2	129.5	11.5	219.5	10.1	196 6
2.....			2 2 3	2 2 5	2 4 2	2 5 5	2 8.4	2 17 1	2 9.4	2 29.1		
3.....					2 58 0	2 136.0	2 62.0	2 224 0	2 74.0	2 327 0		
4.....			2 3.8	2 2 8					2 31 3	2 64.6		
5.....			.6	3.2	4 3	54.0	24.4	320.0	62.9	510.0		
6.....					2 1 4	2 1.7			2 2.1	2 5.1		
Wheat: 1.....			4.0	92 8			6.7	69.4	5 9	89.0		
Corn: 1.....	1.4	1 8	2 7	4.9					3.5	18.8		

<sup>1</sup> Nos. 1, 2, etc., correspond to those numbers of table 2 and refer to the same plants

<sup>2</sup> Green weights; all of the others dry weights

Where plants were grown in solutions of various  $\text{PO}_4$  concentrations the root-top ratio decreased with increasing  $\text{PO}_4$  concentrations in all cases excepting one wheat and one cotton series, which will be discussed later (table 2). Not only was the root-top ratio usually greater at lower  $\text{PO}_4$  concentrations, but the average weight of the roots in some cases (tomatoes (3), cotton (1), and wheat (1), table 3) was greater for some in the lower than in the higher concentrations. The much greater growth of the tops of cotton plants as compared with the roots for increasing  $\text{PO}_4$  concentrations is shown in figures 6 and 7.

These results are just the opposite of those reported by Turner (8) on wheat, barley, and cotton. His solutions, however, were not comparable to those used in this experiment since his low phosphate concentration was not only higher than the highest used in the experiment described above but higher than is commonly found in soil solu-



FIGURE 6.—Cotton plants grown in solution with  $\text{PO}_4$  concentrations of 3.2 (A) and 0.8 p. p. m. (B).

tions. The total concentration of salts in his low  $\text{PO}_4$  solution, moreover, was only a little over half that of his high  $\text{PO}_4$  solution.

The root-top ratio of wheat plants grown at the lowest  $\text{PO}_4$  concentration and that of cotton at the highest concentration in one series appeared to be exceptions to the rule that the root-top ratios decreased with increasing  $\text{PO}_4$  concentration. The wheat plants at this low concentration had finished growing and produced ripe seeds, while those at the two higher concentrations were still producing many new tillers and making rapid vegetative growth. The exceptional cotton plants were making vegetative growth when harvested, while those at the three lower concentrations had apparently stopped growing. A comparison of plants at different stages of growth is probably misleading since, as is shown by the work of Brenchley and Jackson (2), there is, in general, a decrease in the root-top ratio with the age of the plant.

Plants transferred from solutions of adequate  $\text{PO}_4$  concentrations to phosphate-free solutions showed marked increases in root-top ratios. With one exception the amount of phosphate in the solution before the plants were transferred was 1.0 p. p. m. or less, so that the relatively large amount of growth of the roots as compared with that of the tops after transference to phosphate-free solutions could hardly be due to phosphorus stored in the roots. In two cases (wheat (1) and corn (1), table 4) the average weight of the roots of plants transferred to solutions without phosphate was greater than that of the plants allowed to remain in solutions containing phosphate. The results of this group of experiments are shown in table 4.

THE EFFECT OF DIFFERENT PHOSPHATE CONCENTRATIONS ON THE TIME OF MATURITY

Peas, cotton, and wheat were grown in solutions with phosphate concentrations maintained at various levels and observations made as to the time of their maturity. The results of this work (table 5) show that with the exception of cotton at the lowest concentration, at which there was very little growth and no seed matured, the plants at the lower concentrations matured the earliest.

It is commonly accepted that large applications of phosphorus hasten maturity of crops in countries where the growing period is short. Experiments with solution cultures, both as reported in this paper and observations made at other times, have shown that the growing period of plants is shortened where the amount of  $\text{PO}_4$  is low. In the culture solutions all other necessary elements were maintained in adequate amounts so that phosphorus was the only limiting factor. Since under conditions of low fertility in the field unfertilized plants mature earlier than those with adequate fertilization, it seems probable that the depletion of one or more of several of the necessary elements may be responsible for this early maturity. Where maturity is hastened by large applications of phosphorus the effect is therefore probably an indirect one. That is, there is greater growth because of the large applications of  $\text{PO}_4$  and, owing to its more rapid growth, the plant absorbs more rapidly other ions and thus some other element, perhaps nitrogen, becomes a limiting factor causing early maturity.

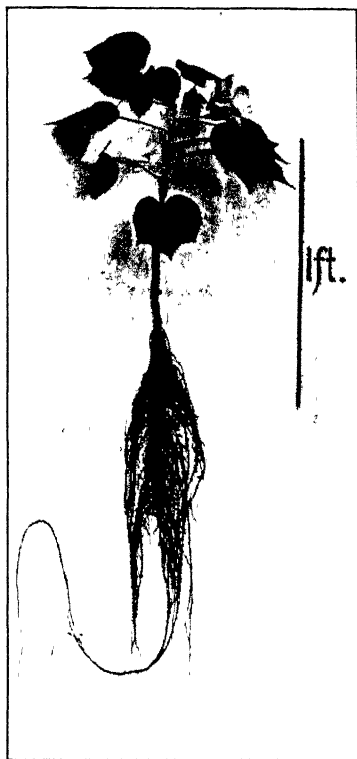


FIGURE 7.—Cotton plant grown in solution with a  $\text{PO}_4$  concentration of 0.2 p. p. m.

TABLE 4.—Average weight of tops and roots and root-top ratio of plants of 4 different kinds when grown in culture solutions of various phosphate concentrations and later transferred to solutions containing and not containing phosphate

Kind of plant and experiment no	Time with $\text{PO}_4$ before transference	$\text{PO}_4$ concentration of culture	After transference to solution—							
			Containing no $\text{PO}_4$				Containing $\text{PO}_4$			
			Weight of roots	Weight of tops	Root-top ratio	Time	Weight of roots	Weight of tops	Root-top ratio	$\text{PO}_4$ concentration of culture
Tomato:	Weeks	P. p. m.	Grams	Grams		Weeks	Grams	Grams		P. p. m.
1.....	7	0.4	0.6	1.5	0.40	3	1.1	5.5	0.20	0.4
2.....	7	.4	1.5	2.7	.55	6	10.7	47.0	.23	.4
3.....	7	.4	2.1	4.5	.47	6	23.0	77.1	.30	.4
4.....	7	.4	12.0	116.0	1.75	3	21.7	173.5	1.30	.4
5.....	7	.4	19.5	121.5	.91	6	1201.2	1647.0	1.31	.4
6.....	4	2.10	16.7	111.6	1.58	2	121.8	140.1	1.54	2.10
7.....	4	2.10	11.4	14.8	1.77	4	148.1	146.1	1.33	1.10
Wheat										
1.....	9	.4	1.8	3.1	.58	3	1.1	7.6	.11	.4
2.....	9	.4	2.6	5.0	.52	6	5.0	22.1	.23	.4
3.....	9	.4	122.4	116.0	1.40	3	133.0	167.0	1.19	.4
4.....	9	.4	125.2	118.2	1.44	6	175.4	1186.4	1.40	.4
5.....	4	.4	19.0	14.7	1.91	4	111.6	119.0	1.61	.4
Corn. 1.....	4	.4	5.2	15.08	.34	6	4.4	23.2	.19	.4
Cotton: 4										
1.....	9	1.040	3.1	27.4	.11	6	6.5	69.0	.09	50
2.....	6	1.040	3.2	25.1	.13	9	6.5	69.0	.09	50

<sup>1</sup> Green weight; all others dry weight

<sup>2</sup>  $\text{PO}_4$  concentration brought up to 1.0 p. p. m. once a week.

<sup>3</sup> Data from an experiment to determine whether cotton, if given large amounts of phosphate during earlier stages of growth, could make normal growth afterward if deprived of phosphate in the culture media.

TABLE 5.—The effect of various phosphate concentrations in the culture solutions on the time of maturity of plants of four different kinds

[The seeds of all plants of each group were germinated and the plants transferred to the experimental solutions and harvested at the same time]

Plant	$\text{PO}_4$ concentration	Average dry weight per plant	Description of plant at time of harvest	Comments
Pea	P. p. m.	Grams		
	0.2	10.7	Dead 1 week; seed mature.	
	.8	41.9	Drying at bottom; some of seed still immature.	
Wheat	3.2	47.6	.....do.....	
	.2	96.4	Beginning to dry; seed mature..	
	.8	76.1	Making rapid growth, many new tillers, heads at all states of growth.	A little less dry than plants at 0.8 p. p. m. Well-filled, mature seeds probably account for the high dry weight as compared with the larger plants at higher $\text{PO}_4$ concentrations.
Cotton	3.2	94.9	.....do.....	Plants injured by aphids when first heads appeared.
	.8	145	Not growing.	{ Plants of this group were set out Aug. 10 and harvested Jan. 2; all had many well-developed bolls, none of which opened, probably due to continued cloudy weather during November and December.
	3.2	231	Making good growth and blooming.	
	12.8	199	.....do.....	
	.2	4	Some green leaves but making no growth.	Four small bolls to 41 plants; bolls did not open.
	.4	54	All leaves off, 96 percent of bolls open.	Average number of bolls per plant, 6.
	.8	320	Most of leaves off; 90 percent of bolls open.	Average number of bolls per plant, 41.
	3.2	510	Some plants still making good growth; 49 percent of bolls open.	Average number of bolls per plant, 74.

## SUMMARY AND CONCLUSIONS

Solution-culture experiments were carried out in a study of the relationship of the  $\text{PO}_4$  concentration of the medium to the type and size of the root system and the time of maturity of certain plants.

Two types of experiments were carried out in an effort to determine whether or not the size and type of root system played a part in limiting the minimum  $\text{PO}_4$  concentration at which certain kinds of plants could make satisfactory growth. In one type of experiment different kinds of plants were grown in solutions with  $\text{PO}_4$  concentrations maintained at 0.1 and 0.8 p. p. m., and a study was made of the relationship of the size and type of root system to the amount of growth. In another type of experiment, cotton, a plant with a root system presenting comparatively small surface for absorption and requiring a relatively high  $\text{PO}_4$  concentration, was grown in containers with buckwheat and tomatoes, plants having more extensive root systems and making better growth at low  $\text{PO}_4$  concentrations. Phosphate concentrations in the solutions were maintained at various levels, and the amount of phosphorus in the part of the stem between the roots and cotyledons and in the leaves was determined. Plants with the larger root systems made better growth at low  $\text{PO}_4$  concentrations and more phosphorus was found in their stems than in the stems of plants with root systems of comparatively small surface.

Two groups of experiments were carried out in a study of the so-called stimulating effect of phosphorus on root development. In one group, plants were grown in solutions in which the  $\text{PO}_4$  concentrations were maintained at various low levels; in the other group plants were first grown with an adequate supply of phosphorus and then transferred to phosphate-free solutions. Comparisons showed that increasing  $\text{PO}_4$  concentrations resulted in decreases of the root-top ratios.

The effect of  $\text{PO}_4$  concentrations on the time of maturity was studied by growing different kinds of plants in solutions in which the  $\text{PO}_4$  concentrations were maintained at various levels. Plants in lower  $\text{PO}_4$  concentrations matured the earliest.

Size and type of root system probably play an important part in determining the minimum  $\text{PO}_4$  concentration at which the different kinds of plants make satisfactory growth.

High concentrations of  $\text{PO}_4$  in solution cultures do not stimulate root development.

Plants growing in culture solutions of low  $\text{PO}_4$  concentrations mature earlier than those grown in solutions of high  $\text{PO}_4$  concentration.

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# THE EFFECTS OF LOW-PHOSPHORUS RATIONS ON GROWING PIGS<sup>1</sup>

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## INTRODUCTION

The discovery that aphosphorosis in domestic animals occurs quite frequently under ordinary feeding conditions in many localities throughout the world, has served to direct the attention of nutrition workers to the role of phosphorus in animal nutrition. As a result, many studies have been made; in most of them cattle or sheep were used as experimental animals. Among such studies are those of Theiler (9, 10);<sup>3</sup> Malan, Green, and du Toit (7); and Eckles and his associates (4, 5). Considerable experimental work (4, 8) has dealt with the effect of phosphorus-deficient rations on animals and with the correction of these deficiencies. The literature reports rather fully the effects of low-phosphorus rations on other species of animals, but not on swine.

However, Bethke and his associates (1) have described the effects of the calcium-phosphorus relationship of the ration on the growth and bone formation in the pig, and Dunlop (3) has described the importance of calcium and phosphorus in the ration, as well as the relationship between these two elements with and without vitamin D.

Otherwise the influence of low-phosphorus rations on body growth and on the mineral composition of the blood and bones has been little discussed; and no specific information, so far as the authors are aware, has been recorded regarding other effects on growing pigs, of phosphorus-deficient rations in which the calcium and other essential nutrients were supplied in adequate amounts. The effects of low-phosphorus rations on growing pigs, observed during the progress of experiments designed primarily to show the phosphorus requirements for growing swine, are here presented. The results of two feeding tests are recorded in which young pigs were fed rations differing only in phosphorus content.

## EXPERIMENTAL PROCEDURE

The basal ration employed in these experiments consisted of 74 percent of pearl hominy, 10 percent of tapioca roots, 10 percent of blood meal, 4 percent of alfalfa-leaf meal, 1.5 percent of dried brewers' yeast, 0.5 percent of iodized salt, and 5 cc of cod-liver oil per pig per day. Enough calcium carbonate was added to these ingredients to bring the calcium content up to 0.77 percent in the first experiment and up to 0.70 percent in the second.

<sup>1</sup> Received for publication July 9, 1935; issued March 1936. This paper represents a portion of a thesis presented by C. E. Aubel in partial fulfillment of the requirements for the degree of doctor of philosophy at the University of Minnesota in 1935. Contribution no. 114 of the department of animal husbandry, no. 190 of the department of chemistry, and no. 62 of the department of veterinary medicine.

<sup>2</sup> The authors wish to give credit to Prof. J. F. Merrill for making the chemical analyses of the feeds and to R. R. Roepke for the analyses of the blood. Credit is also due Prof. D. L. Mackintosh for assistance in slaughtering.

<sup>3</sup> Reference is made by number (italic) to Literature Cited, p. 159.



Three lots of six pigs each were used in each of the two experiments, which were carried on for 24 weeks each. The experiments were started shortly after the pigs were weaned at about 9 weeks of age, when they weighed from 40 to 50 pounds each. All the lots were housed in a well-lighted and well-ventilated building provided with individual pens paved with concrete and measuring 8 by 8 feet. Each pen had a 10- by 8-foot outside exercise area open to the south, paved with concrete and fenced with wire. This outside area allowed the pigs access to the direct rays of the sun and exposed them, to a considerable extent, to ultra-violet rays.

All the animals were fed individually twice a day. The amount fed daily was changed from time to time, but each received the amount of feed that the one consuming the least feed would eat morning and evening. This arrangement insured all the pigs ingesting the same amount of feed.

The pigs were weighed and measured every 28 days throughout the experiments. An analysis of the calcium and inorganic phosphorus in the blood was made at the beginning of each experiment and at the end of each 28 days. A single sample of blood was drawn from the tail for these determinations. The Youngburg and Youngburg (11) method was used for the determination of inorganic phosphorus, and the calcium was determined by the Clark and Collip (2) method. The progressive effects of the low-phosphorus rations on the development of the bones, carcasses, and internal organs were noted at the end of each 56 days, when two animals from each lot were slaughtered.

In order to feed the different lots of pigs at different levels of phosphorus, monocalcium phosphate was incorporated in the basal ration to bring the phosphorus content up to the desired level. In the first experiment the phosphorus content of the ration fed lot 1 was 0.15 percent, that of the ration fed lot 2, 0.29 percent, and that of the ration fed lot 3, 0.59 per cent. The experimental pigs were fed phosphorus at these levels for 7 weeks, at the end of which time a second mixture was made, which, because of variations in the phosphorus content of the ingredients, contained slightly higher amounts of phosphorus. The amounts were 0.18 percent in that fed to lot 1, 0.33 percent in that fed to lot 2, and 0.59 percent in that fed to lot 3. Rations containing phosphorus at these levels were fed during the remaining 17 weeks of the experiment.

In the second experiment the phosphorus content of the ration of lot 4 was 0.15 percent; that of the ration of lot 5, 0.23 percent; and that of the ration of lot 6, 0.30 percent. Enough feed containing these amounts of phosphorus to supply the pigs during the entire 24 weeks of feeding was mixed before the experiment began.

#### EXPERIMENTAL DATA

In the course of these experiments five very decided effects of feeding the pigs the low-phosphorus rations were observed. These were: (1) The effect on the appetite; (2) the effect on the utilization of feed and storage of energy; (3) the effect on growth of the body and the development of bone and muscle; (4) the effect on the inorganic phosphorus in the blood; (5) the effect on the consumption of water and the excretion of urine.

## EFFECT ON THE APPETITE

Loss of appetite in the low-phosphorus pigs in lots 1 and 4 began to appear as early as the fifth week after the pigs were placed on feed. This made it necessary to feed very carefully in order to have them consume the same amounts of feed as the other pigs. By the end of the fifth month it was no longer possible to get these pigs to eat the ration in a dry form. It was possible, however, because of an increased thirst which they developed, to induce them to eat their feed by mixing it with the drinking water. Toward the end of the experiments it was necessary to withhold all drinking water except that mixed with the feed. By mixing small portions of the feed in the water five or six times each day, by the end of the experiments it was possible to induce the pigs to consume nearly 6 pounds of feed daily. So greatly did they crave water that they soon learned to wait for the feed to settle, and it was necessary for an attendant to keep stirring the mixture in order to have the feed consumed.

No difficulty over loss of appetite was experienced with the pigs in lots 2, 3, 5, and 6, which were on higher levels of phosphorus.

TABLE 1.—Average feed consumed daily per pig, daily gains in weight, and feed required per 100 pounds gain in weight by 28-day periods in experiments 1 and 2

## EXPERIMENT 1

Period (28 days) no	Pigs <sup>1</sup>	Feed consumed daily			Average daily gain in weight			Feed required per 100 pounds gain in weight		
		Lot 1	Lot 2	Lot 3	Lot 1	Lot 2	Lot 3	Lot 1	Lot 2	Lot 3
	Number	Pounds	Pounds	Pounds	Pounds	Pounds	Pounds	Pounds	Pounds	Pounds
1.....	6	2.5	2.5	2.5	0.72	0.81	0.85	347.2	308.6	294.1
2.....	6	3.1	3.1	3.1	.74	.96	.99	418.9	322.9	313.1
3.....	4	3.9	3.9	3.9	1.06	1.09	1.16	367.9	357.7	336.1
4.....	4	4.5	4.5	4.5	1.15	1.26	1.20	391.3	357.1	375.0
5.....	2	5.5	5.9	5.9	.74	1.41	1.31	743.2	418.4	450.3
6.....	2	5.7	5.9	5.9	1.28	1.50	1.48	445.3	393.3	398.6
Weighted average.....	-----	3.7	3.7	3.7	.90	1.07	1.08	417.1	344.6	341.0

## EXPERIMENT 2

		Lots 4, 5, and 6	Lot 4	Lot 5	Lot 6	Lot 4	Lot 5	Lot 6
	Number	Pounds	Pounds	Pounds	Pounds	Pounds	Pounds	Pounds
1.....	6	2.7	0.65	0.83	0.91	415.3	325.3	296.7
2.....	6	3.3	.81	1.00	1.06	407.4	340.0	311.3
3.....	4	3.5	.86	1.05	1.19	406.9	333.3	294.1
4.....	4	4.4	.87	1.34	1.41	505.7	328.3	312.0
5.....	2	5.7	1.41	1.61	1.62	404.2	354.0	351.8
6.....	2	6.0	1.05	1.28	1.27	571.4	468.7	472.4
Weighted average.....	-----	3.7	.85	1.09	1.16	439.1	342.6	321.7

<sup>1</sup> Of the original 6 pigs, 2 were slaughtered at the end of the second period, 2 at the end of the fourth, and 2 at the end of the sixth

## EFFECT ON THE UTILIZATION OF FEED AND THE STORAGE OF ENERGY

That the pigs on the low-phosphorus diet did not utilize their feed as well as those on the higher levels of phosphorus is shown in table 1. The data show that lots 1 and 4, the low-phosphorus groups, had during each period of the experiment the largest feed consumption

per 100 pounds gain. In lot 1, experiment 1, the consumption per 100 pounds gain fluctuated from period to period but was exceptionally high in period 5. As a rule, the fluctuation varied as the average daily gains, but the very high requirement of feed in period 5 was accompanied by a low average daily gain. No explanation can be offered for this unless the sudden change from a dry-feeding method to a thin-slop method might have caused some irregularity in body weight. In lot 4, experiment 2, in which the change was made gradually from dry to slop feeding when the pigs began to lose their appetites, no such fluctuation was observed.

While no direct measurements of the storage of energy were made in these experiments, on the basis of gains in weight it is evident that the pigs receiving the low-phosphorus ration stored less energy than those receiving higher amounts. The difference, however, is not so great as the difference in the body weights would indicate, because the low-phosphorus-fed pigs carried a higher percentage of fat and showed better finished carcasses.

The thickness of the back fat opposite the seventh, eleventh, and thirteenth dorsal vertebrae was measured in experiment 2. The results, shown in table 2, indicate, according to the fat index suggested by Hankins and Ellis (6), that there was an average of approximately 96 pounds of fat on the carcasses of the low-phosphorus pigs, as compared with 108 pounds on those of the high-phosphorus pigs in lots 5 and 6.

TABLE 2.—Average thickness of the back fat at the seventh, eleventh, and thirteenth ribs of two pigs in lots 4, 5, and 6 at the end of the fourth and sixth periods of experiment 2

Period (28 days) no.	Thickness at seventh vertebra			Thickness at eleventh vertebra			Thickness at thirteenth vertebra		
	Lot 4	Lot 5	Lot 6	Lot 4	Lot 5	Lot 6	Lot 4	Lot 5	Lot 6
4.....	Mm 34	Mm 27	Mm 30	Mm 30	Mm 26	Mm 27	Mm 42	Mm 39	Mm 35
6.....	51	43	45	46	40	41			

In the case of dairy animals on a low-phosphorus ration, it is impossible to keep the animal fat, for not only is the feed poorly utilized but it is impossible to induce the animal to eat sufficient amounts of the ration to store energy during the later stages of aphosphorosis. It was possible with the pigs in this study to take advantage of their increased thirst to force them to eat their feed in the form of a slop, and thus provide excess energy to be deposited in the form of fat, even though they were not utilizing their feed as fully as the normal pigs.

#### EFFECT ON THE GROWTH OF THE BODY AND THE DEVELOPMENT OF BONE AND MUSCLE

One of the more marked effects of the lack of phosphorus in the ration was a failure of the body to grow normally, and especially to develop bones and muscle to a normal extent.

It can be seen from the lower graphs in figure 1 that the hogs in experiment 1 receiving the low-phosphorus ration (lot 1), although

they received the same amount of feed and had the same initial weight, were about 17 percent lighter at the end of the experiment than those in lots 2 and 3. In experiment 2 there was a difference of about 25 percent in the weight of the pigs receiving a low-phosphorus ration (lot 4) and that of those on the higher phosphorus levels (lots 5 and 6).

Not only did the pigs on the low-phosphorus level weigh less (fig. 1, *C*, *D*), but their lengths (fig. 1, *A* and *B*, and fig. 2) and heights (figs. 1, *A*, *B*) were also less.

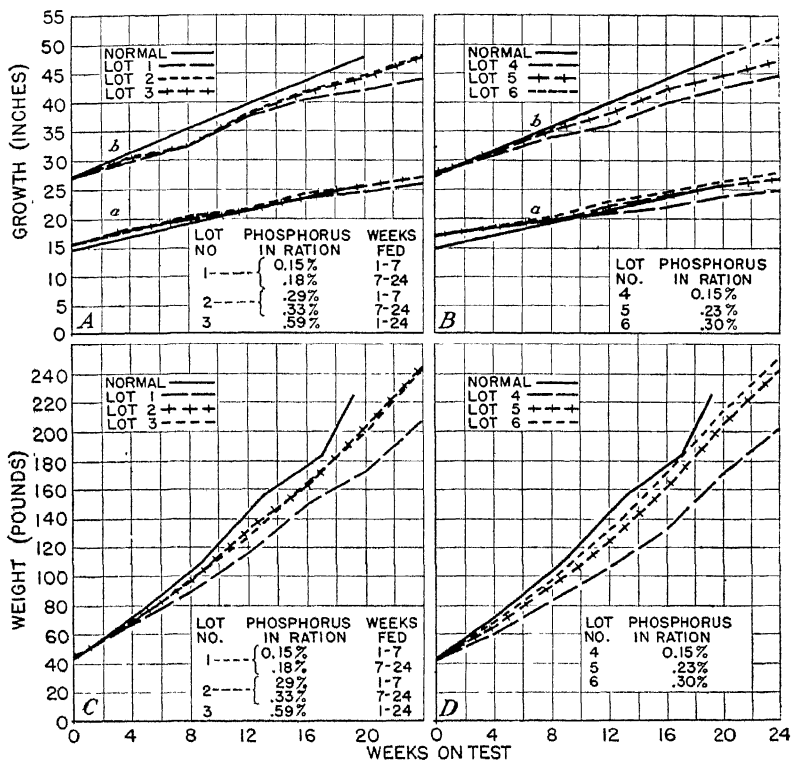


FIGURE 1.—Increases in the dimensions and weights of pigs on low-, medium-, and high-phosphorus intakes as compared with the dimensions and weights of normal pigs. Curves for normal animals were drawn from unpublished data supplied by the Division of Animal Husbandry of the University of Minnesota and represent the average of the records of 134 pigs of record-of-performance litters. The pigs in these litters were of all breeds and were self-fed on excellent rations. *A* and *B* show at *a*, height at shoulder, and at *b*, length from base of ear to root of tail; *C* and *D* show body weights. Lots 1 and 4 were on low-phosphorus, lots 2 and 5 on medium-phosphorus, and lots 3 and 6 on high-phosphorus rations, as explained in the text.

The lighter body weight was also correlated with poorly developed bone, as is shown in table 3. This table shows for pigs on the different levels of phosphorus, the variations in the bones, the specific gravity, breaking pressure, dimensions of the bone, and the ash content.

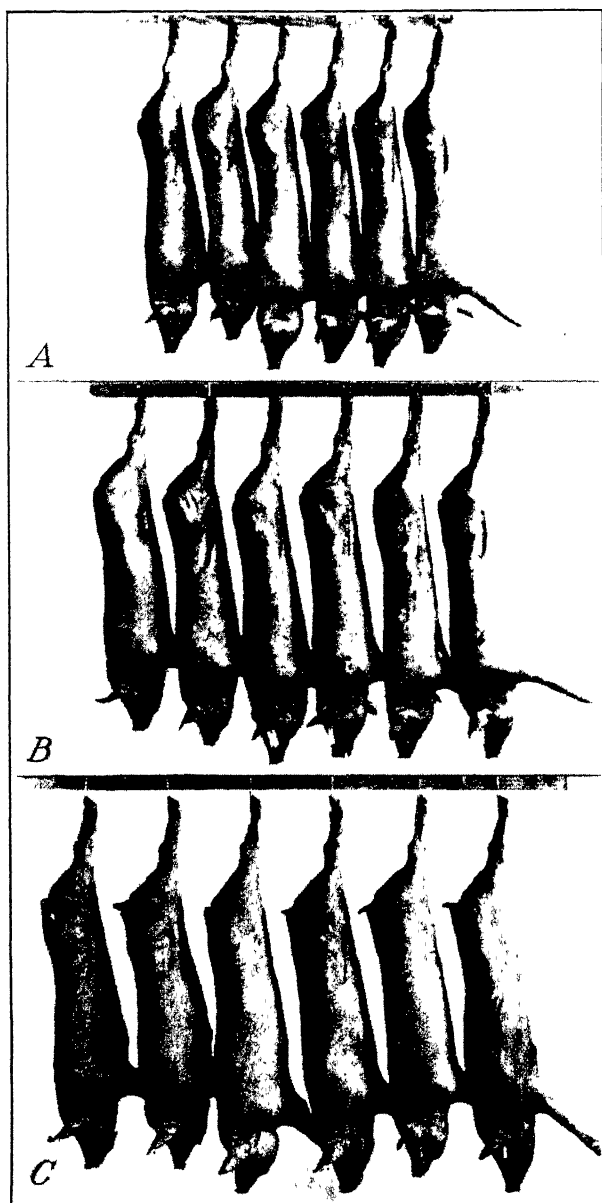


FIGURE 2—Carcasses showing the development of the pigs in experiment 1. *A*, At the end of 8 weeks; *B*, at the end of 16 weeks; *C*, at the end of the experiment. Note the length and thickness of the carcasses. The two carcasses on the left in each group are from lot 1 (low-phosphorus), the next two carcasses in each group are from lot 2 (medium-phosphorus); and the two on the right in each group are from lot 3 (high-phosphorus).

TABLE 3.—Average specific gravity, breaking strength, length, diameter, thickness of wall, and ash of the green leg and rib bones of two pigs in each lot, by 56-day periods in experiments 1 and 2

## EXPERIMENT 1

Period (28 days) no.	Bones	Specific gravity			Breaking pressure			Length of bones <sup>1</sup>			Diameter of bones <sup>2</sup>			Thickness of wall <sup>3</sup>			Ash <sup>4</sup>				
		Lot 1	Lot 2	Lot 3	Lot 1	Lot 2	Lot 3	Lot 1	Lot 2	Lot 3	Lot 1	Lot 2	Lot 3	Lot 1	Lot 2	Lot 3	Lot 1	Lot 2	Lot 3		
2	Humerus	1.102	1.241	1.224	Pounds	305	575	Pounds	5.49	5.57	Inches	0.660	0.713	Inches	0.094	0.133	Inches	48.14	57.35	Percent	59.64
	Femur	1.098	1.223	1.222	190	595	590	6.31	6.52	6.46	0.636	0.647	0.725	0.103	0.153	169	57.35	60.03	60.32		
	Sixth rib	1.076	1.200	1.170	25	78	79	5.33	5.62	5.67							53.08	59.63	61.08		
	Eleventh rib	1.091	1.226	1.155	16	64	65	5.18	5.47	5.58							52.29	58.80	60.97		
	Humerus	1.124	1.221	1.237	420	745	910	6.57	6.78	6.67	7.44	8.25	8.25	1.20	1.68	184	52.72	59.63	61.08		
4	Femur	1.108	1.225	1.219	427	945	975	7.03	7.73	7.81	7.45	8.22	7.97	1.15	1.91	187	52.29	58.80	60.97		
	Sixth rib	1.042	1.159	1.193	61	157	157	6.67	6.97	7.16							54.16	62.29	62.82		
	Eleventh rib	1.071	1.216	1.211	50	156	153	6.48	7.00	7.02	8.52	9.92	8.77	1.11	2.06	182	52.88	61.04	61.84		
	Humerus	1.167	1.339	1.366	500	1,435	1,380	6.95	7.32	7.14	8.95	9.87	8.77	0.86	2.06	179					
	Femur	1.167	1.338	1.345	585	1,490	1,455	7.72	8.41	8.23	8.05										
6	Sixth rib	1.121	1.398	1.395	60	370	390	7.52	7.72	7.71											
	Eleventh rib	1.193	1.483	1.455	55	345	345	5.53	7.73	7.62											

## EXPERIMENT 2

Period (28) days) no.	Bones	Lot 4	Lot 5	Lot 6	Lot 4	Lot 5	Lot 6	Lot 4	Lot 5	Lot 6	Lot 4	Lot 5	Lot 6	Lot 4	Lot 5	Lot 6
		Lot 4	Lot 5	Lot 6	Lot 4	Lot 5	Lot 6	Lot 4	Lot 5	Lot 6	Lot 4	Lot 5	Lot 6	Lot 4	Lot 5	Lot 6
2	Humerus	1.135	1.177	1.182	320	470	480	5.82	5.67	5.66	0.660	0.667	0.706	0.092	0.103	0.102
	Femur	1.122	1.164	1.171	330	540	550	6.51	6.46	6.36	0.660	0.663	0.712	0.087	0.191	0.135
	Sixth rib	1.079	1.116	1.166	29	75	78	6.24	5.96	6.11						
	Eleventh rib	1.077	1.147	1.188	29	48	47	6.01	6.12	6.12						
	Humerus	1.173	1.205	1.184	440	755	897	6.85	6.65	6.90	7.48	8.36	8.83	107	172	165
4	Femur	1.166	1.191	1.242	375	747	900	6.70	7.70	7.90	7.32	7.89	7.97	0.90	152	175
	Sixth rib	1.128	1.177	1.252	56	114	176	6.50	7.50	7.60						
	Eleventh rib	1.196	1.204	1.318	46	86	154	7.20	7.38	7.45						
	Humerus	1.181	1.221	1.300	560	840	1,220	7.17	7.38	7.68	8.24	8.86	9.32	136	176	103
	Femur	1.171	1.221	1.294	580	1,030	1,310	7.72	8.46	8.80	8.00	8.92	9.11	0.130	157	180
6	Sixth rib	1.079	1.177	1.284	65	160	295	7.17	8.08	8.48						
	Eleventh rib	1.152	1.281	1.308	62	145	295	6.92	7.79	7.89						

<sup>1</sup> Length over all of leg bones, ribs shortest distance from head to end of shaft.<sup>2</sup> Average of smallest and greatest diameter at breaking point.<sup>3</sup> Average of smallest and greatest thickness of wall at breaking point.<sup>4</sup> Average of the 2 leg or 2 rib bones.<sup>5</sup> Determinations lost in the burning of the chemistry building

The ribs and vertebrae of the pigs fed low levels of phosphorus showed poor calcification similar to that usually observed in animals suffering from low-phosphorus rickets. The poor development of bone is also indicated by the fact that as early as 7 weeks after the beginning of the experiments the legs of the low-phosphorus pigs were somewhat deformed. This condition became more marked as the experiments progressed, so that by the end of the experiments the pigs had difficulty in moving about in their pen.

It is rather to be expected that the growth of bone, which contains a high percentage of calcium and phosphorus, would be materially influenced if the phosphorus were not provided in the feed. It is not generally thought, however, that this mineral plays such an important part in the development of the muscle, because the percentage of ash in muscle is comparatively low. When it is considered that phosphoric acid is an integral part of the nuclei of the cells, which have much to do with the cell development, and that the phospholipins play an essential part in the function of the muscle fiber, it does not seem impossible that a deficiency in phosphorus would be reflected in decreased muscle development.

#### EFFECT ON THE INORGANIC PHOSPHORUS OF THE BLOOD

The results of the effect of low-phosphorus rations on the inorganic phosphorus of the blood (table 4) are in accord with the results reported by other investigators for swine.

TABLE 4.—Average calcium and inorganic-phosphorus content of the blood of pigs by 28-day periods in experiments 1 and 2

EXPERIMENT 1							
Period no.	Pigs	Calcium (per 100 cc of serum)			Inorganic phosphorus (per 100 cc of whole blood)		
		Lot 1	Lot 2	Lot 3	Lot 1	Lot 2	Lot 3
	Number	Milli-grams	Milli-grams	Milli-grams	Milli-grams	Milli-grams	Milli-grams
Initial <sup>1</sup> .....	6	11.9	11.9	11.7	4.3	5.5	6.6
1.....	6	12.8	12.3	11.1	3.9	4.9	6.1
2.....	6	13.3	12.4	11.8	3.4	4.8	5.4
3.....	4	13.7	12.9	12.3	3.7	4.8	5.9
4.....	4	14.9	12.8	12.1	2.9	4.8	6.1
5.....	2	13.3	12.1	12.4	3.2	5.1	6.0
6.....	2	12.9	12.1	11.6	3.3	5.8	6.8

EXPERIMENT 2							
Period no.	Pigs	Lot 4	Lot 5	Lot 6	Lot 4	Lot 5	Lot 6
		Milli-grams	Milli-grams	Milli-grams	Milli-grams	Milli-grams	Milli-grams
Initial.....	6	9.7	10.0	10.0	5.5	5.7	5.7
1.....	6	12.4	11.2	11.2	4.1	5.0	5.7
2.....	6	12.6	12.0	12.1	3.4	4.7	5.5
3.....	4	11.0	10.6	10.3	2.9	4.7	5.5
4.....	4	11.9	12.1	11.0	2.7	4.8	5.3
5.....	2	11.5	12.0	11.1	3.5	5.1	5.5
6.....	2	13.2	13.7	11.5	2.6	5.4	6.2

<sup>1</sup> The blood samples for this analysis were taken about 2 hours after the second feed of the experimental ration had been given the pigs.

An inspection of these data clearly shows that the pigs in lots 1 and 4 quickly developed a state of phosphorus deficiency, which became more marked as the experiment progressed. Lots 1 and 4 had the least amount of inorganic phosphorus in the blood. Lots 3 and 6 had the highest percentage, while the amount in lots 2 and 5 were between that of the other two lots. Since each lot received a different level of phosphorus in its ration and since each lot had a distinctly separate level of inorganic phosphorus in the blood, it is clearly evident that the amount of phosphorus in the feed readily reflects itself in the blood.

This fact is shown further by the initial analysis of the blood of the pigs in experiment 1 (table 4). Through an oversight at the time of starting the experiment, blood was not drawn for analysis until after the second feed of the experimental ration had been consumed. In this short time the amount of phosphorus in the two feeds affected the inorganic phosphorus of the blood of the pigs in the three lots, so that three distinct levels resulted, corresponding to three definite levels in the rations.

#### EFFECT ON THE CONSUMPTION OF WATER AND THE EXCRETION OF URINE

The literature, so far as the authors have observed, does not mention an increased thirst as one of the symptoms of aphosphorosis. However, the data for a digestion trial with dairy cows reported by Riddell, Hughes, and Fitch (8), in which the consumption of water was accurately measured, showed that the average amount of water consumed by the two low-phosphorus cows was about 4 percent more than consumed by the cows receiving the same ration supplemented with phosphorus. The low-phosphorus cows also excreted 27.2 percent more urine. However, no attention was called to these variations in the discussion of this digestion trial.

In experiment 1 it was noticed as early as the second week that the pigs of lot 1 were consuming considerably more water than the pigs in the other lots receiving a higher level of phosphorus. Accordingly, a check of the consumption of water of the pigs was made for 2 days of each month. The results are shown in table 5.

TABLE 5.—*Water consumed daily by and weight of kidneys of pigs in experiment 1*

Period (28 days) no.	Pigs	Water consumed daily <sup>1</sup>			Weight of kidneys <sup>2</sup>		
		Lot 1	Lot 2	Lot 3	Lot 1	Lot 2	Lot 3
	Number	Kilograms	Kilograms	Kilograms	Grams	Grams	Grams
1.....	6	4.77	3.68	3.31			
2.....	6	9.09	7.13	5.59	220	177	185
3.....	4	8.95	8.22	6.63			
4.....	4	11.18	10.13	8.27	302	264	230
5.....	2	(3)	12.31	11.45			
6.....	2	(3)	15.86	14.13	395	280	275

<sup>1</sup> Average per pig calculated from the water consumption for 2 days of each month.

<sup>2</sup> Average for 2 pigs in each lot.

<sup>3</sup> No data were secured for lot 1 for periods 5 and 6.



While it was apparent that more urine was being excreted, no measure was made of it in this experiment.

Because the pigs exhibited excessive thirst and urination, special attention was given to the weight and appearance of the kidneys at the time of slaughter. Table 5 shows that the kidneys of the pigs on the low-phosphorus ration were larger than those from the pigs receiving the higher levels of phosphorus. It is also apparent that the differences in the weights of the kidneys became more marked as the experiment progressed.

During the second experiment it was possible, by placing the pigs in a metabolism crate, to measure not only the water consumption but the excretion of urine for a 3-day period.

The water consumption was 11,514, 6,190, and 5,903 g, and the urinary excretion was 8,541, 2,300, and 2,355 g for lots 4, 5, and 6, respectively. The average weights of the kidneys for lot 4 was 411 g, for lot 5, 366 g, and for lot 6, 335 g.

Thus the increased thirst and the increased urinary excretion of the pigs on the low-phosphorus level were evident again. The kidneys also were larger in the low-phosphorus-fed pigs at the close of this experiment.

Histological examination of the enlarged kidneys from the lots of pigs maintained on the low level of phosphorus disclosed that they were distinctly enlarged and light in color, presenting the appearance of a "large white kidney." Microscopically this organ showed evidence of a chronic diffuse nephritis of the parenchymatous type, and presented widened glomerular spaces around the glomerular tufts and also widely distended uriniferous tubules with flattened epithelial cells. A granular debris was present in some of the tubules.

It is significant that the impaired kidney corresponded with the excessive urination and accompanying thirst observed in these experiments.

#### SUMMARY

The results are reported of an investigation of the effects of the feeding of different levels of phosphorus in the rations of 36 young pigs.

Two experiments, each involving three lots of six pigs each, were carried on for 24 weeks.

Data are presented to show the effect of deficient amounts of phosphorus in the ration on the growth and development of pigs.

The results obtained indicate that the abnormalities resulting from feeding low-phosphorus rations were: (1) A loss of appetite, (2) a poor utilization of feed and storage of energy, (3) a failure to make normal growth and to develop bone and muscle normally, (4) a lowering of inorganic phosphorus in the blood, and (5) a marked increase in thirst and a corresponding excretion of urine.

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## LIFE HISTORY OF AGAMERMIS DECAUDATA, A NEMATODE PARASITE OF GRASSHOPPERS AND OTHER INSECTS<sup>1</sup>

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### INTRODUCTION

Of the various nematodes that parasitize insects, those belonging to the family Mermithidae probably occur in the greatest variety of these hosts. As the presence of a mermithid parasite usually has a marked influence on the development of an insect, frequently retarding or modifying growth, usually inducing sterility, and finally resulting in death, these nematodes are of considerable interest from an economic standpoint. Reports from various parts of the world indicate that mermithids are an important factor in holding certain pestiferous insects in check, at least in some localities. As mermithids are usually long, slender animals they are sometimes referred to as "hairworms." Unfortunately this term has also been used for members of the Gordiidae, a group of animals whose resemblance to mermithids is largely superficial and with which they should not be confused.

In spite of the fact that mermithids have long been known to zoologists and numerous references to them are found in zoological literature, information regarding them is rather meager. In some instances writers have merely reported their occurrence and given a brief morphological description which is frequently so inadequate as to leave the reader in doubt whether the organism in question was a mermithid or a gordiid. However, numerous contributions, including the papers by Rauther (13)<sup>2</sup>, Hagmeier (9), and Steiner (16, 17, 18, 19), have contributed considerable information pertaining to the morphology and taxonomy of the group. In regard to life histories our knowledge is much less satisfactory. Although studies on this phase of the subject were made as early as 1853 by Von Siebold (15) and Meissner (12) and later by Corti (5), it was not until 1905 that Kohn (11), working with *Paramermis contorta* (Linstow, 1889) Kohn, 1905, a parasite of *Chironomus* larvae, presented the first comprehensive life-history study. Further observations on this species were made by Comas (4).

Judging from available information, it appears that the typical mermithid life cycle consists of a parasitic larval development in the

<sup>1</sup> Received for publication Sept. 9, 1935, issued March 1936. Thesis presented to the graduate council of the George Washington University in partial fulfillment of the requirements for the degree of doctor of philosophy.

<sup>2</sup> Reference is made by number (italic) to Literature Cited, p. 197.

body cavity of the host, followed by a free-living adult stage usually passed in the soil or in mud and sand underlying streams and lakes. *Tetradonema plicans* Cobb, 1919, which infests the larvae of *Sciara coprophila* Lintner, is evidently an exception, as it apparently passes its entire life as a parasite (10). This should probably be regarded as a specialized type of life cycle wherein the free-living stage has been eliminated.

In the north-central and northeastern parts of the United States, over an area extending southward to northern Virginia and westward to eastern Nebraska, the more common species of grasshoppers harbor mermithid parasites which consist largely of two species, *Agamermis decaudata* Cobb, Steiner, and Christie, 1923 (3), and *Mermis subnigrescens* Cobb, 1926. The evident economic significance of these parasites of grasshoppers has been noted by several investigators. I. G. McGrue, of Audubon, Minn., is reported by Riley (14) as asserting that "in that part of the country the hairworms destroyed as many locusts in 1875 as did any other enemy." Writing in 1900, Forbes (7, p. 129) makes the following comment:

Long thread-like, milk-white hair-worms (*Mermis*) are often found in the abdomens of grasshoppers, living there as internal parasites, and escaping after maturity to enter the earth, where they pass the winter, pair, and produce myriads of eggs the following spring. The young from these infest the grasshoppers of the year and assist greatly in the reduction of any excess of numbers.

In August 1908 a grasshopper outbreak at Columbia Cross Roads, Pa., was investigated by J. A. Hyslop, of the Bureau of Entomology, United States Department of Agriculture. When specimens of *Melanoplus femur-rubrum* DeGeer, the species responsible for the outbreak, were dissected, cream-white worms, about 3 or 4 inches in length, were taken from the bodies of the insects. In one case 12 worms were taken from a female *M. femur-rubrum*, 10 of which were 3 inches long. Hyslop observed that "the infested grasshoppers act sluggish" and that "the genital organs (ovaries and testes) were always consumed where worms were present."<sup>3</sup>

Glaser and Wilcox (8), while investigating a grasshopper epidemic near Dummerston Station, Vt., in 1917, noted a high mortality among these insects. This mortality was due to a mermithid parasite which infested nearly 30 percent of all the grasshoppers in that locality at the time, *Melanoplus atlantis* Riley and *M. bivittatus* Say being the most common species. Between 1923 and 1925 there occurred in Door County, Wis., a grasshopper outbreak of considerable severity, the principal species being *Camnula pellucida* Scudd. The writer visited this region during August 1925 and found the grasshoppers heavily infested with mermithid parasites. There is little doubt that these parasites were an important factor in terminating the plague.

The parasite involved in the grasshopper outbreak in Door County, Wis., was *Mermis subnigrescens* and that found by Glaser and Wilcox at Dummerston Station, Vt., was probably the same species. The parasite found by Hyslop at Columbia Cross Roads, Pa., was probably *Agamermis decaudata*, a species which is abundant in northeastern Virginia. It is the species with which the present paper deals.

For convenience and clarity in description, the life cycle of *Agamermis decaudata* is divided into five stages, as follows: (1) The

<sup>3</sup> HYSLOP, J. A. Unpublished note

embryonic and ovic larval stage, which includes the development of the embryo from the time the egg is deposited until the larva emerges; (2) the preparasitic larval stage, which includes the period between hatching and the entrance of the larva into a host; (3) the parasitic larval stage, which includes the development within the host; (4) the postparasitic larval stage, which extends from the time the larva emerges from the host until it undergoes its final molt; and (5) the adult stage.

## EMBRYONIC AND OVIC LARVAL STAGE

### DEPOSITION, STRUCTURE, AND SIZE OF THE EGGS

The adults of *Agameremis decaudata* are found coiled within small cavities in the soil, usually about 5 to 15 cm below the surface. Here eggs are deposited and undergo embryonic development, becoming plastered over the parent nemas and over the walls of their earthen cavities. It is estimated that an average female during her life will deposit about 10,000 eggs.

The egg when first deposited (fig. 1, A) is somewhat discoidal, having the form of a sphere considerably flattened on opposite poles. As ordinarily seen under the microscope it presents a circular outline measuring, on an average, about  $160\mu$  in diameter. When viewed on edge it is ellipsoidal in outline, the length of the short axis being about two-thirds that of the long axis. The protoplasmic mass is surrounded by a thin membrane. Outside of this, in the case of *Hexameremis albicans* (Siebold, 1848) Steiner, 1924, Meissner (12) reported the existence of a colorless transparent layer composed of a semifluid substance which he called the albuminous layer (Eiweisschicht). Enclosing the whole is an outer membrane or "shell",  $1.5\mu$  to  $2\mu$  thick and of a pale amber or straw color. The color, at first very faint, darkens as the egg develops.

Adult females of *Agameremis decaudata* collected by the writer varied in length from 3 to 46.5 cm. It was noted that eggs deposited by the smaller females averaged about  $120\mu$  in diameter (measured on the longer axis) as against an average of  $163\mu$  for those deposited by larger females. Results from a study of the length of females and the size of eggs deposited by them are shown in table 1.

TABLE 1.—Relation between length of female *Agameremis decaudata* and size of eggs

Length of female (cm)	Diameter of eggs			Length of female (cm)	Diameter of eggs		
	Mini- mum	Maxi- mum	Aver- age		Mini- mum	Maxi- mum	Aver- age
3.0.....	$\mu$ 121	$\mu$ 130	$\mu$ 128.9	25.0.....	$\mu$ 159	$\mu$ 174	$\mu$ 166.3
3.2.....	110	118	113.0	27.0.....	150	170	163.2
3.5.....	110	118	114.0	28.0.....	160	178	167.8
4.5.....	106	120	110.4	28.0.....	164	172	168.9
5.0.....	110	125	116.8	30.0.....	150	179	161.3
6.0.....	125	133	129.0	31.0.....	155	176	166.5
6.0.....	123	127	125.4	33.0.....	147	164	156.0
8.0.....	103	137	119.2	34.0.....	144	180	160.1
9.5.....	120	137	129.1	35.0.....	146	160	153.6
12.0.....	115	141	125.2	36.0.....	144	172	166.0
15.0.....	154	182	169.7	45.0.....	162	168	166.0
18.0.....	154	176	166.9	46.5.....	164	170	167.5
24.0.....	141	164	153.5				

The data in table 1 show that the size of the eggs deposited does not increase gradually as the length of the female increases. By comparing the average measurements of the eggs deposited by each female it will be observed that those deposited by females 3 to 12 cm long comprise one group while those deposited by females 15 to

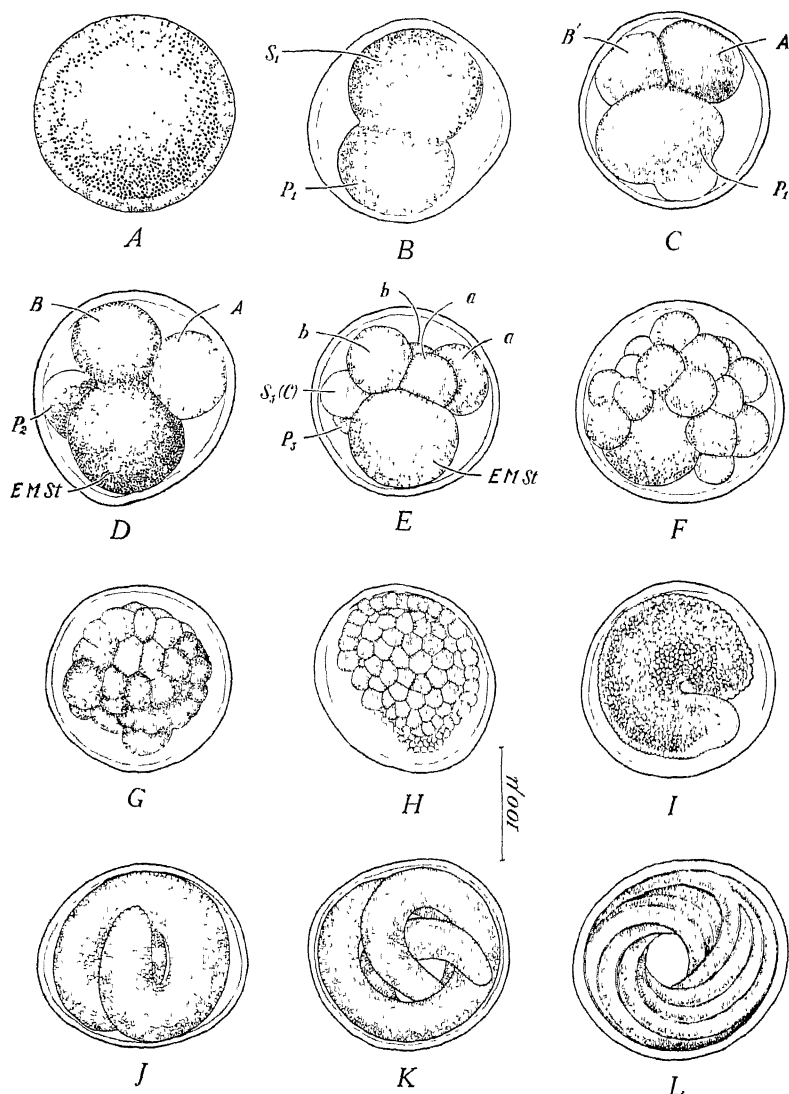


FIGURE 1—Egg of *Agamermis decandata*: A, At time of deposition, B, after about 24 hours, C, after about 30 hours, D, after 36 to 40 hours; E, after 4 to 5 days; F, after 6 to 7 days, G, after 8 to 9 days, H, after 10 to 11 days, I, after 13 to 15 days, J, after 16 to 18 days; K, after 20 to 24 days, L, after 35 to 40 days.  $S_1$ , First somatic stem cell;  $P_1$ , undifferentiated cell;  $A'$ , anterior primary ectoderm cell;  $B'$ , dorsal ectoderm cell;  $P_2$ , second undifferentiated cell;  $EMSt$ , endoderm-mesoderm-stomodaeum cell;  $S_2(C)$ , posterior dorsal ectoderm stem cell,  $a, a, b, b$ , cells of primary ectoderm.

46.5 cm long comprise another group. If the variations in the size of the eggs be represented by a curve, this curve will be "two-peaked" with but a slight overlapping at the base. At first one is inclined to suspect the existence of two species. Such information as we possess, however, indicates that there is but one species, although the point is not yet settled. The matter will be discussed further in connection with the parasitic larva.

#### CLEAVAGE

During the first 10 to 12 hours after deposition, the central protoplasmic mass contracts slightly until there exists between it and the outer shell a clear space  $30\mu$  to  $35\mu$  in thickness. The first cleavage is perpendicular to the flattened poles of the egg and results in two cells slightly unequal in size. This division is usually complete at the end of 24 to 30 hours after the egg is deposited. The larger of the two blastomeres (fig. 1, *B*, *S*<sub>1</sub>), is the first to divide, resulting in two cells about equal in size. The remaining blastomere of the first pair (fig. 1, *C*, *P*<sub>1</sub>), now divides. On the side opposite the two small blastomeres now present (*A'* and *B'*), a projection of protoplasm is pushed out and is eventually split off. The cell thus formed (fig. 1, *D*, *P*<sub>2</sub>), is smaller than any of the others and differs from them in appearance, being composed of a somewhat clearer protoplasm. This cleavage is usually complete at the end of about 40 hours and leaves the egg in a four-celled stage. Cell *P*<sub>2</sub> divides, resulting in two blastomeres, *P*<sub>3</sub> and *S*<sub>3</sub> (*C*), each of which again divides. In the meantime the derivatives of the larger of the first two blastomeres (*A'* and *B'*) continue in their cleavage, producing a cluster of cells which tends to grow over and envelop the large blastomere (*EMSt*). This latter undergoes 2 or 3 cleavages before it is completely hidden, each division resulting in daughter cells about equal in size. At the end of 8 to 9 days the young embryo has developed to the stage shown in figure 1, *G*. In 16 to 18 days an embryo of about one and a half coils (fig. 1, *J*) is produced, and after this the number of coils increases rapidly. At first there is a tendency for the coils to assume an irregular, crisscross arrangement, and 20- to 30-day embryos are likely to be in this condition; but after about 30 days the coils arrange themselves near the periphery of the disk (fig. 1, *L*).

#### DEVELOPMENT OF THE STYLET

In the head region of a 20-day ovic larva the outline of the developing esophagus (fig. 2, *A*, *oe*) can usually be seen. Posteriorly it becomes increasingly indistinct, disappearing about  $40\mu$  from the anterior end. The rounded anterior contour is more difficult to see than the walls which bound it laterally. About  $16\mu$  from the anterior end of the larva the esophagus is constricted, thus forming a differentiated anterior part (fig. 2, *A*, *oe ant*). Back of the constriction the esophagus has about the same diameter as the part anterior to the constriction. Near the center of the anterior part the primordium of the odontium or tooth can be seen (fig. 2, *A*, *vesic*). It is a pyriform vesicle about  $8\mu$  long by  $3\mu$  wide, and in a living specimen appears somewhat lighter in color than the surrounding tissue.

In a 25-day ovic larva the esophagus is still a prominent feature, the anterior part having enlarged to a diameter four-fifths as great



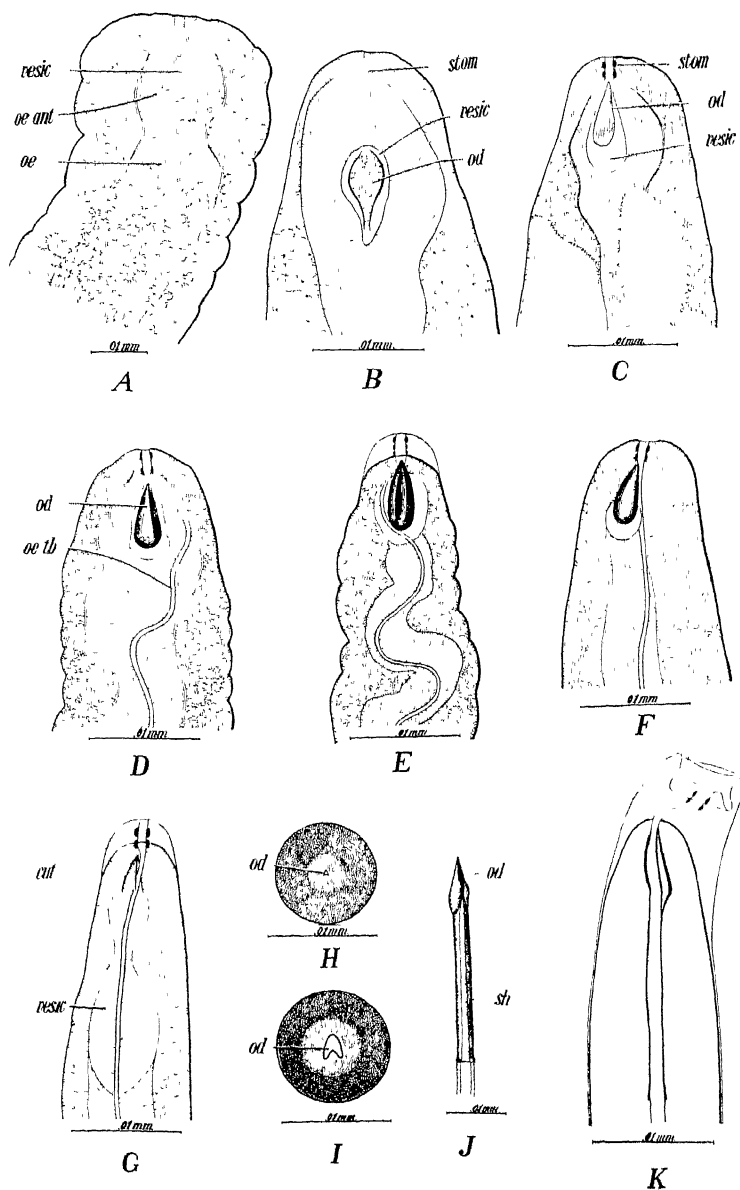


FIGURE 2—Development of stylet in *Agamermis decaudata*. A, 20-day larva, lateral view; B, 25-day larva, lateral view; C, 28-day larva, lateral view; D, 30-day larva, lateral view; E, 35-day larva, lateral view; F, 40-day larva, lateral view; G, 45-day larva, lateral view; H, 30-day larva, cross section through middle of odontostyl "head"; I, 30-day larva, cross section through tip of odontostyl; J, fully developed odontostyl, semidiagrammatic; K, molt at end of 55 days, lateral view. stom, Stoma; cut, cuticle; oe, esophagus; oe ant, anterior enlargement of esophagus; oe tb, esophageal tube; od, odontium; hd, "head" of odontostyl; sh, "shaft" of odontostyl; resic, vesicle.

as the diameter of the larva in that region. The vesicle (fig. 2, *B*, *vesic*) is about twice as long as wide and has a diameter one-third as great as the diameter of the esophageal enlargement. In the center of the vesicle and nearly filling it is a spindle-shaped mass of rather dense substance, bounded laterally by heavily cuticularized walls (fig. 2, *B*, *od*).

In an average 28-day ovic larva the esophagus and the anterior esophageal enlargement are both more clearly defined than in preceding stages. The developing odontium (fig. 2, *C*, *od*) now has the shape of an elongated cone with a pointed anterior end and a hemispherical base. It has moved slightly anteriorly and is enclosed in the vesicle, which is about one-third larger and may somewhat resemble it in shape. The wall of the stoma is reinforced by 2 or sometimes 3 hooplike (?) thickenings which when seen in lateral aspect present the appearance shown in figure 2, *C*. The tubular lumen of the esophagus may sometimes be seen at about this stage of development.

The average 30-day ovic larva shows few changes from a 28-day larva. On the ventral side of the developing odontium (fig. 2, *D*, *od*), a groove extends from near the point to near the base. This groove may best be understood by studying a front view of the head, where the odontium appears crescent-shaped in optical cross section (fig. 2, *H*, *L*, *od*). A tortuous, heavy-walled tube now occurs within the esophagus, extending past the vesicle and terminating near the base of the stoma, with which it may possibly communicate, although the opening was not seen.

In a 40-day ovic larva (fig. 2, *F*) the walls of the esophagus are more difficult to observe, and as the larva develops they become increasingly obscure. The stoma now communicates directly with the esophageal tube, which runs straight back without the windings characteristic of earlier stages. The developing odontium, which up to this time has occupied a more or less axial position, now moves toward the dorsal side, the groove lying adjacent to the esophageal tube. The vesicle often encompasses only the base of the odontium and in some cases seems to have disappeared. At about this time a second vesicle (fig. 2, *G*, *vesic*) is formed, oblong in shape and encompassing that portion of the esophageal tube which is destined to form the shaft of the stylet. The wall of this part of the tube now becomes thicker. At the end of about 50 days the stylet (fig. 2, *J*, *K*) is completely formed. When the odontium is united with the shaft the anterior extremity of the esophageal tube lies in a groove of the odontium (fig. 2, *G*), with which it is eventually joined. The odontium, therefore, acts as a reinforcement which is wrapped around and united with the anterior end of the shaft.

For a stylet formed from a modified tooth or odontium or from an odontium together with a portion of the esophageal tube, as in the present instance, the term "odontostyl" is proposed. Such stylets also occur in the genus *Dorylaimus* and related genera. For a stylet formed through a modification of the entire stoma or buccal cavity the term "stomatostyl" is proposed. Such stylets occur in the genera *Anguillulina* (= *Tylenchus*) and *Applenchoides*.

## TWENTY-DAY OVIC LARVA

At the end of 20 days the larva usually forms about two and one-half loops within the egg shell (fig. 1, *K*). The anterior end of such a larva, for a distance of  $30\mu$  or  $40\mu$ , is filled with a comparatively clear, finely granular tissue in which distinct cell walls were not seen. As already noted, it is in this clear anterior region that the developing esophagus is visible. Back of this region the body is filled with spherical globules which vary from  $1.5\mu$  to  $3\mu$  in diameter. In situ these globules tend to be polygonal because of the pressure of one upon another, but when broken from the body they become spherical. They are probably fat or oil globules and contain a clear nongranular substance that darkens with osmic acid.

## THIRTY-DAY OVIC LARVA

At the end of 30 days the larva (fig. 3) has attained a length nearly equal to that of the preparasitic larva. There is a cylindrical stoma  $3\mu$  to  $4\mu$  long by  $2\mu$  wide, its wall reinforced by the hooplike (?) thickenings already mentioned. Whether these extend entirely around the stoma was not determined, but from a study of the molted lining they seemed to be more strongly developed dorsally and ventrally. In lateral view they appear as 2 or 3 pairs of dark spots (fig. 2, *G*). As the diameter of the esophageal tube is less than that of the stoma, the latter is narrowed somewhat where it joins the former. This reduction in width takes place on the dorsal side, causing in profile view a slightly asymmetrical appearance.

The anterior portion of the esophagus is well differentiated; it is a subcylindrical structure  $210\mu^4$  in length and possesses three noticeable swellings or enlargements. The first (fig. 3, *oe enl* (1)) is at the anterior end and contains the developing odontium. Posteriorly the diameter decreases rather gradually to about  $6\mu$  to  $7\mu$ . The second enlargement (fig. 3, *oe enl* (2)) is slightly anterior to the nerve ring. Here the width increases gradually to about  $12\mu$ , then decreases to  $4\mu$  or  $5\mu$ . This width is retained for a distance of about  $80\mu$  and then again increases to  $10\mu$  or  $12\mu$  to form the posterior enlargement or bulb (fig. 3, *bb*). The bulb is partly obscured by granules and globules, which fill the body from this point posteriad.

A distinct esophageal tube,  $0.5\mu$  to  $1\mu$  in diameter, occurs within the esophagus throughout this anterior part. Embedded in the dorsal wall of the esophageal enlargement at the nerve ring and with a diameter two-thirds as great as that of the enlargement itself, is a large, clavate cell (fig. 3, *cl*). The wider end of the cell is directed cephalad and the narrower end extends through the nerve ring. A spherical nucleus is located near the anterior end of the cell. As seen in lateral aspect this cell is  $30\mu$  long by  $8\mu$  wide. Extending caudad from the esophageal bulb for a distance of  $200\mu$ , the posterior portion of the esophagus (fig. 3, *post oe*) can be faintly seen. It is nearly cylindrical, has a diameter two-thirds as great as the corresponding body diameter, and is packed with granules or globules that are smaller and different in appearance from those that surround and partly obscure it. This structure probably includes not only the esophagus proper but the primordia of the glands and other organs later associated with it, although they cannot now be distinguished.

<sup>4</sup> Measurements given in the morphological descriptions were derived from 3 or 4 specimens selected as being of about average size.

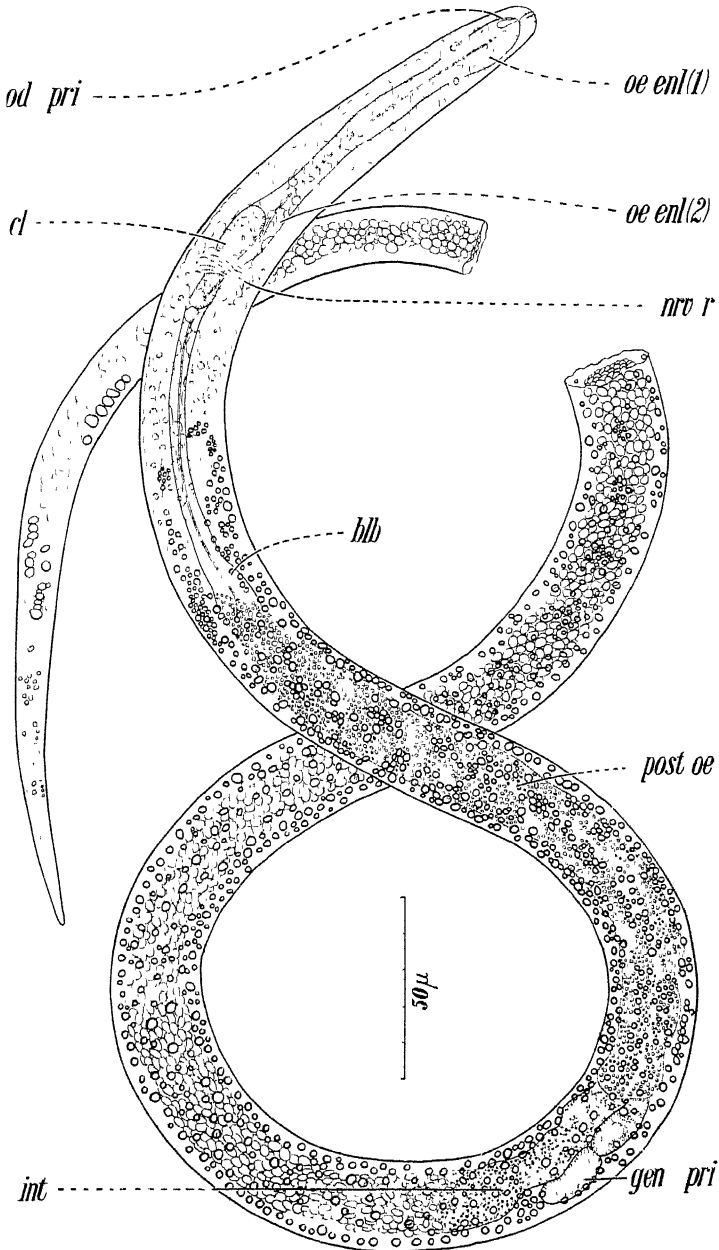


FIGURE 3.—Ovic larva of *Agamermis decaudata* 30 days after deposition of egg: *blb*, Bulblike enlargement of esophagus; *cl*, cell (?); *gen pri*, genital primordium; *int*, intestine; *od pri*, primordium of "head" of odontostyl; *oe enl (1)*, anterior enlargement of esophagus; *oe enl (2)*, middle enlargement of esophagus; *nrv r*, nerve ring; *post oe*, posterior part of esophagus.

Back of the esophagus the developing intestine (fig. 3, *int*),  $50\mu$  long, is faintly visible. On the ventral side, at the junction of the intestine and esophagus, is located the primordium of the reproductive system (fig. 3, *gen pri*). It is apparently composed of two cells, each of which is  $15\mu$  to  $20\mu$  long by  $6\mu$  to  $8\mu$  wide. Caudad from the posterior end of the intestine the body is filled with a continuous cylindrical mass of closely packed granules and globules.

#### FORTY-DAY OVIC LARVA

At the end of 40 days the ovic larva (fig. 4) is very similar in structure to the preparasitic larva. The esophagus is composed of an anterior and posterior part. The anterior part is possibly homologous to the corpus and the posterior part to the isthmus and bulb as found in certain other nematodes. In the anterior part the swellings noted in the 30-day larva have disappeared, and there remains a cylindrical structure  $4\mu$  in diameter containing a conspicuous tube  $1.5\mu$  to  $2\mu$  in diameter. At about  $220\mu$  from the cephalic end of the animal the esophagus enlarges to a width of  $10\mu$  and at the point where this enlargement begins the character of the lumen is abruptly altered. This change is from a tube more or less circular in cross section to one with a triradiate cross section and marks the junction of the anterior and posterior parts of the esophagus. The posterior part has a length of about  $300\mu$ . Its anterior end has the form of a bulblike swelling (fig. 4, *blb*)  $35\mu$  long by  $10\mu$  wide. The remaining position,  $245\mu$  in length, while perhaps structurally similar to the bulblike swelling, is greatly modified in shape by the presence of three large glands. The largest of these glands (fig. 4, *subd oe gl*) is right subdorsal in position. It first appears in about 35 days as a subcylindrical structure extending anteriorly from the base of the esophagus about two-thirds the distance to the bulblike enlargement. In a 40-day larva it has increased considerably in diameter, extends farther anteriorly, and often appears to have a branched anterior end. It is filled with closely packed, nearly spherical globules averaging about  $3\mu$  in diameter. At its anterior end a mass of similar globules often forms a separate body, the size and shape of which differ in different specimens. On the left subventral side there are eventually developed two smaller glands. These, however, are usually not discernible at this stage and will be discussed in connection with the preparasitic larva. The excretory pore is opposite the nerve ring. The pore itself was not seen, but the duct leading to it can usually be detected.

The genital primordium (fig. 4, *gen pri*) remains about the same as in a 30-day larva. The intestine has increased slightly in length, but otherwise it has changed little. The granules at the posterior end of the intestine are arranged in transverse rows (fig. 4, *nd*), preparatory to the formation of the node, a structure to be described later.

When a 30- to 40-day larva is removed from the egg sometimes the cuticle is seen separating from the body at the two extremities. This is the beginning of the first molt. As molting progresses the cuticle is frequently invaginated at the anterior end because of the lining of the esophagus (fig. 2, *K*). The cuticle finally breaks at some point on the body, and the anterior portion is pushed off over the head and the posterior portion over the tail. In an egg containing an almost

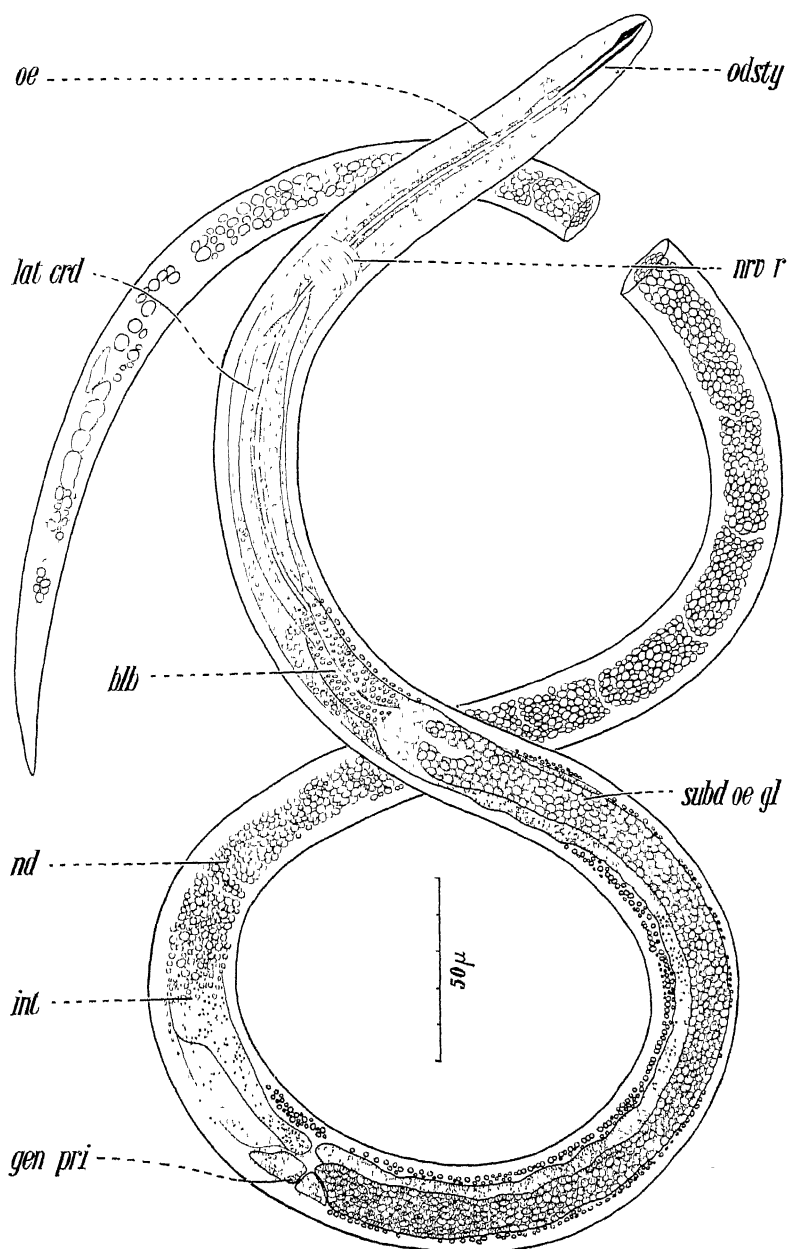


FIGURE 4.—Ovic larva of *Agamermis decaudata* 40 days after deposition of egg. *bulb*, Bulblike enlargement of esophagus; *gen pri*, genital primordium; *int*, intestine; *lat crd*, lateral cord; *nd*, node; *nrv r*, nerve ring; *oe*, esophagus; *odsty*, odontostyl; *subd oe gl*, subdorsal esophageal gland.

fully developed ovic larva the cast cuticle can usually be seen wound into a ball and located near the center of the egg. At a temperature of 12° to 25° C., molting begins within 1 to 2 months. The lining of the stoma and of the esophageal tube, as well as the rings(?) reinforcing the wall of the former, are shed with the old cuticle. Sometimes the outer cuticle breaks from the lining of the esophageal tube and stoma, and the latter is left protruding from the mouth. In one instance of this kind the ejection of this lining was completed while the specimen was under observation. The length of the ejected lining was equal to the distance from the anterior end to a point opposite the genital primordium, indicating that this entire region of the alimentary tract is cuticular-lined stomodaeum.

#### TIME OF HATCHING

At the beginning of winter each adult female is surrounded by the eggs produced by her during the preceding summer. All stages of development are represented, many eggs containing fully developed ovic larvae. While an occasional egg will hatch during the winter, hatching, for the most part, is suppressed until the advent of warm spring weather.

On November 27, 1924, three females were dug from the soil in such a manner that the eggs surrounding them were not disturbed. Each female was placed in a container devised for the purpose, and reburied. When first examined on February 4, 1925, some of the eggs had already hatched, and in every container a few larvae were found in the soil near the eggs. On April 22 larvae could not be found around any of the egg masses, but on May 5 three active larvae were seen in one of the containers. On May 19, active larvae were fairly numerous in all three containers. On June 2, active larvae were present but not so numerous as on May 19. On June 21, active larvae were again fairly numerous. However, the egg masses had not been noticeably depleted and the number that had hatched up to this time represented but a very small percentage of the total number of eggs present on November 27. On July 12, the soil in one of the containers was teeming with active larvae and nearly all of the eggs had hatched. In the other two containers the eggs had all hatched and the larvae had apparently migrated, for none was found.

From the foregoing observations it appears that, in the region of Falls Church, Va., in spite of a limited amount of hatching during winter and early spring, the greater part of the larvae emerge between late June and the middle of July.

#### PREPARASITIC LARVAL STAGE

##### STRUCTURE OF THE PREPARASITIC LARVA

Ovic larvae, when kept at room temperature, appear to be fully developed within 50 to 60 days. However, they do not leave the egg immediately. In nature, as already noted, a larva which becomes fully developed during late summer will ordinarily remain within the egg until the following spring.

Preparasitic larvae (fig. 5) vary in length from 4.42 to 5.59 mm, the majority being from 5 to 5.10 mm. This does not include individuals hatched from the smaller type of eggs previously referred to, the taxonomic status of which will be discussed later. The width of the

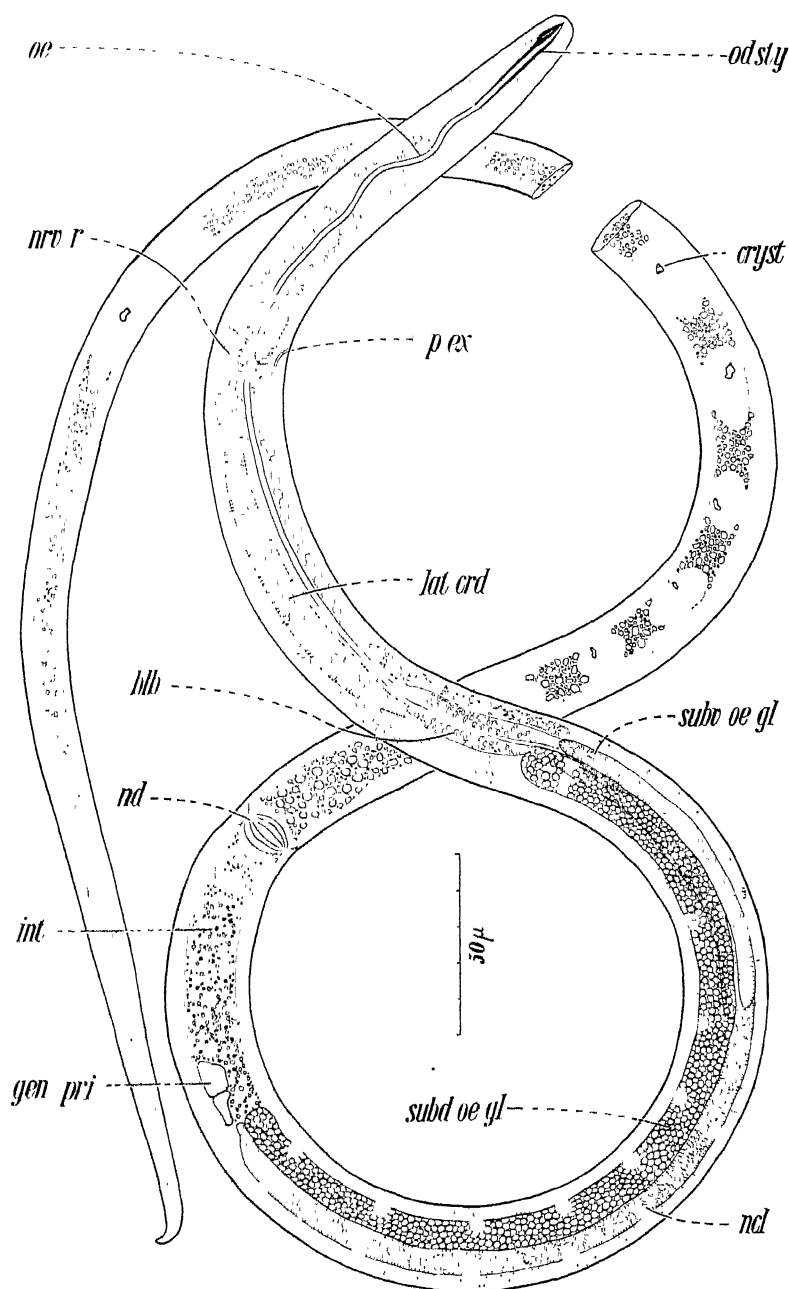


FIGURE 5.—Preparasitic larva of *Agamermis decaudata*: *hbb*, Bulblike enlargement of esophagus; *cryst*, crystals, probably of excretory materials; *gen pri*, genital primordium; *int*, intestine; *lat crd*, lateral cord; *ncl*, nuclei of primordia of stichocytes; *nrv r*, nerve ring; *oe*, esophagus; *nd*, node; *odsty*, odontostyl; *p ex*, excretory pore; *subd oe gl*, subdorsal esophageal gland; *subv oe gl*, subventral esophageal gland.



body is as follows: At the base of the spear,  $16\mu$  to  $17\mu$ ; at the nerve ring,  $20\mu$  to  $22\mu$ ; at the esophageal bulb,  $22\mu$  to  $24\mu$ ; at the base of the esophagus (region of the genital primordium),  $23\mu$  to  $25\mu$ ; slightly posterior to the node,  $20\mu$  to  $22\mu$ . The body is divided into an anterior region, which contains the principal organs, and a posterior region, which is a food-storage region and a propelling organ. These two body regions are separated by the node (fig. 5, *nd*), where an automatic amputation takes place when the larva enters its host. The cuticle is  $1.5\mu$  to  $2\mu$  thick and appears smooth. If it is transversely striated the striae are exceedingly difficult of resolution. The head is rounded and bears six small papillae. The amphidial openings are situated  $6.5\mu$  from the anterior extremity. In carmine-stained specimens the internal amphidial structure is conspicuous, extending posterior to a point  $4\mu$  distant, where it seems to end abruptly, the stain not having penetrated farther. The odontostyl (fig. 5, *odsty*) is from  $33\mu$  to  $37\mu$  long and is composed of an enlarged subconical pointed head  $8\mu$  long by  $3\mu$  wide and a posterior shaft  $2\mu$  wide and very slightly expanded at its base. The entire structure is hollow, with the anterior opening on the ventral side. The odontostyl is surrounded by well-developed muscles.

The anterior portion of the esophagus is about the same as that already described for a 45-day ovic larva. Transverse sections through the cuticularized esophageal tube show that a thin layer of tissue surrounds the tube, although the total diameter of the esophagus in this region does not exceed  $4\mu$ . While in general the tube is circular, in transverse section its shape is often suggestive of a triradiate origin. In parasitic larvae the triradiate character of this part of the lumen is sometimes very apparent.

About  $245\mu$  from the anterior end of the body the esophagus widens to form a bulblike enlargement (fig. 5, *blb*)  $10\mu$  in diameter, which possesses distinct radiating muscles. As previously noted, the lumen changes abruptly in character at the anterior end of the bulb. The posterior portion of the esophagus is partly obscured by the esophageal glands, which extend nearly to the posterior margin of the bulb. The anterior end of the largest gland (fig. 5, *subd oe gl*) lies about  $35\mu$  behind the anterior end of the bulb, and the gland extends caudad the entire length of the esophagus, a distance of about  $270\mu$ . It is right subdorsal in position and is packed with clear, spherical globules  $1\mu$  to  $1.5\mu$  in diameter. There is a region at its anterior end which is somewhat different in appearance and either contains a small group of globules somewhat separated from the others, or lacks globules and is filled with a homogeneous nongranular substance. The two smaller esophageal glands (fig. 5, *subv oe gl*) are left subventral in position and are filled with a homogeneous substance of a granular appearance. Their anterior ends lie slightly behind the anterior ends of the subdorsal gland. They extend caudad  $90\mu$  to  $100\mu$  and at their widest point have a diameter of  $6\mu$  to  $8\mu$ . A nucleus is situated near the anterior end of each. These glands communicate with the lumen of the esophagus through pores. The esophagus proper lies between these glands and is slightly compressed by them. In cross section it appears as a somewhat triangular structure, almost axial in position, with the lumen near its center. Sixteen rather conspicuous nuclei are found in this region of the body. Posteriorly they have a tendency to be arranged in pairs, one on each side of

the esophagus, although their position varies somewhat in different specimens. They are spaced more or less equidistant and the first pair lies slightly in front of the posterior end of the subventral glands. They are nuclei in the primordia of the so-called "stichocytes."

The intestine (fig. 5, *int*) is  $80\mu$  to  $90\mu$  long and extends from the base of the esophagus to the node. It is without a distinct lumen and contains two kinds of granules arranged in a loose network. The smaller and more numerous of these granules do not stain intra-vitam with neutral red, while the larger granules stain promptly. In the anterior two-thirds of the intestine, cell walls could not be seen, but in the posterior part definite transverse walls are present, becoming more conspicuous near the node. A varying number of prominent, slightly concave, transverse septa mark the position of the node. One or sometimes several of the most posterior septa have their convex surface posterior, while the others have their convex surface anterior, and between these there is a small clear space. It is at this point that the amputation of the caudal portion of the body takes place; the septa remaining on the body proper form a plug, which prevents the body contents from exuding. The part posterior to the node constitutes about 80 percent of the total length of the larva. The width back of the node,  $20\mu$  to  $22\mu$ , is slightly less than that in front of the node, and the caudal portion retains this width throughout two-thirds of its length. It then gradually tapers posteriorly, ending in a pointed terminus, which may sometimes be curved at the tip. Except for a terminal portion of about  $200\mu$ , it is filled with a moniliform series of about 50 cells. Each cell has a central nucleus surrounded by a network of protoplasm containing spherical refractive globules up to  $5\mu$  in diameter and smaller granules of  $1\mu$  or less. The cells are separated by a clear space containing 1, or occasionally 2, refractive, irregular-shaped crystals.

In a 25- to 30-day ovic larva the entire caudal portion, except for a short terminal region, is packed with spherical globules forming a continuous cylinder. In about 40 to 45 days these globules become so arranged as to form short cylindrical "segments", each of which is separated from those adjacent by a narrow space. Back of the node these segments are  $20\mu$  to  $30\mu$  long and increase in length posteriorly until they become  $70\mu$  to  $80\mu$  long near the posterior extremity. As the larva develops, the clear spaces separating the segments increase in size, and at the time of hatching each usually contains a small crystal (fig. 5, *cryst*). As the larva becomes older the globules decrease in number and after a few days have nearly all disappeared. At the same time the clear spaces grow larger, and there is a slight increase in the size of the crystals. These globules, which do not stain intra-vitam with neutral red, are probably reserve material which is gradually utilized. The contents of the clear spaces, including the crystals, are interpreted as waste products of metabolism. Among the globules are smaller granules which stain deeply with neutral red, in this respect resembling similar granules in the intestine.

#### MIGRATION TO THE HOST

After hatching, the preparasitic larvae must migrate to the surface of the soil to locate their insect hosts. Although *Melanoplus femurrubrum*, like many Acridiidae, deposit their eggs in the soil, the writer

regards it as improbable that nymphs become infested before leaving the soil. Such a possibility is precluded in the case of *Orchelimum vulgare* Harris and many other tettigoniids by the fact that eggs are deposited in the stems of plants. Observations made both on grasshoppers in the field and on those hatched and reared in the laboratory indicate that nymphs of *M. femur-rubrum* do not often come into actual contact with the soil, but tend to feed and rest on grass and other vegetation. Observations made at night revealed nymphs of this and other species of grasshopper resting on the plants and not on the ground. It appears necessary that the parasitic larvae climb the grass and other vegetation in order to reach their hosts. As drying is fatal, this climbing must be done either at night or early in the morning while the grass is wet with dew, or during rainy weather. That the larvae will climb a moist surface has been demonstrated in the laboratory, where they were found ascending the glass sides of an insect cage.

#### METHOD OF ENTERING THE HOST

Although the writer has never observed the entrance of the parasitic larva into the host in nature, experimental infestation of insects in the laboratory is easily accomplished. As the eggs of both *Agamer-mis decaudata* and *Melanoplus femur-rubrum* hatch readily when brought into the laboratory, an abundance of young individuals of both host and parasite may be had throughout the winter for experimental purposes. To infest a grasshopper nymph with *A. decaudata* the following method was employed and proved satisfactory.

A glass slide was placed on a turntable and with a small candle a wax ring (fig. 6, *a*) about one-fourth of an inch in diameter was run on. With a scalpel a narrow channel was made through this ring at two points (*b*, *c*), in which was laid a long hair. One end of the hair was sealed down with a drop of hot wax (*d*), which at the same time closed that channel. The nymph was wet by being shaken in a vial containing water. This not only rendered the nymph less active and easier to handle but facilitated the entrance of the larvae. With a moistened camel's-hair brush, the nymph was transferred to the slide and placed on its side within the ring. The hair was drawn across its body just back of the pronotum. The free end of the hair was then sealed down with a drop of wax, which at the same time closed the other channel. A nymph thus pinioned may be held securely for a long time without injury. The nymph should rest in a drop of water in which are placed active preparasitic larvae.

The larvae swim rapidly at first, moving in an apparently aimless way, often following the periphery of the drop, and are not noticeably attracted by the presence of the insect. After a few minutes some may be seen with their heads pushed against the nymph or inserted between the segments. Entrance does not necessarily follow this act as the larvae may move or be kicked away and resume their apparently aimless swimming. Soon, however, detached caudal ends will usually appear, and if the detached anterior ends cannot be seen it may be assumed that infestation has taken place. The activity of the nymph often breaks larvae at the node before they have had an opportunity to enter. The detached anterior ends continue active movement and have been seen attempting to bore their way into the nymph, but

hampered by the loss of their propelling mechanism, probably never succeed. The larvae appear to attack the nymph at any point without preference, and have on occasion been seen boring at the distal end

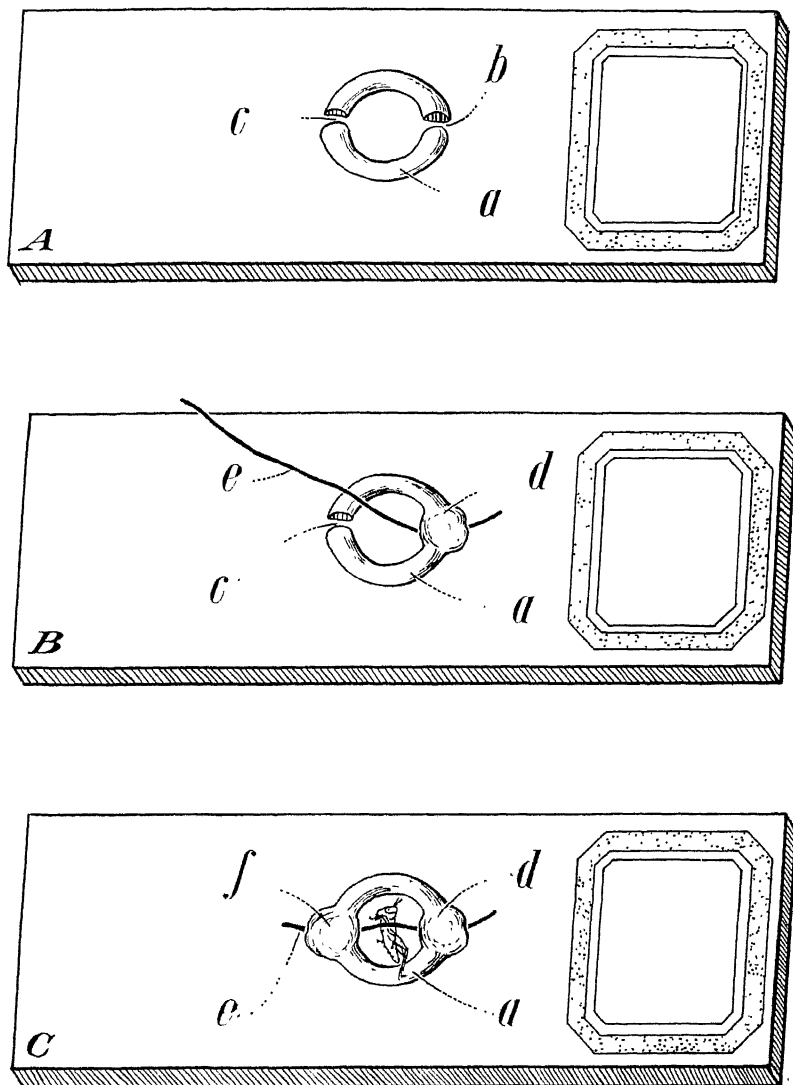


FIGURE 6.—Device with which grasshopper nymphs were experimentally infested: A, Glass slide with wax ring (a) divided at two points (b, c); B, wax ring (a) closed at one point by drop of wax (d) in which is embedded end of hair (e), C, wax ring (a) closed by second drop of wax (f), with nymph fastened in center by hair (e).

of the posterior femur. They seem able to penetrate more easily through the thorax, and probably the majority enter near this region. Despite the fact that the entire operation can be kept under the closest scrutiny, it is exceedingly difficult actually to see the larvae go in.

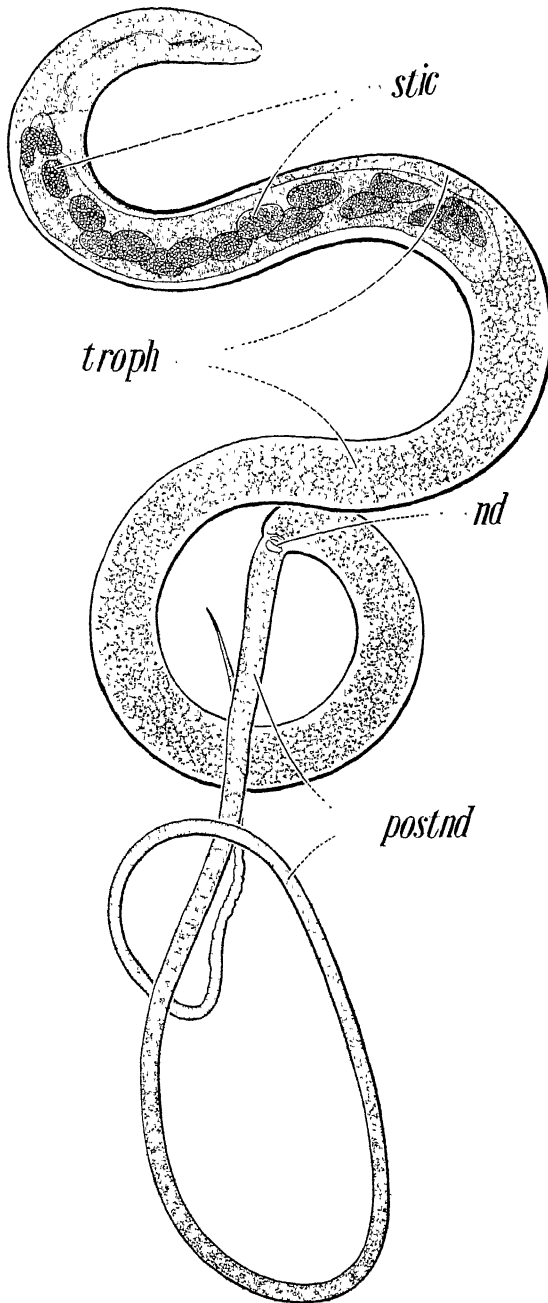


FIGURE 7.—Parasitic larva of *Agamermis decaudata*, about 6 to 8 days in the host, in which the postnodal portion of the body was not detached when the larva entered the host: *nd*, Node; *postnd*, postnodal region of body; *stic*, stichocytes, *troph*, trophosome (modified intestine).

Because of their minute size and the high magnification necessary to follow their movements carefully, they are lost from view immediately they come into contact with the nymph. In only a few cases, where light conditions were exceptionally favorable, were larvae seen in the process of entering. The time necessary for making the puncture varies considerably. Sometimes they penetrate within 1 to 2 minutes and at other times they bore for 5 to 10 minutes. Once the head is inserted they enter immediately.

At this time they usually break at the node, leaving the posterior portion of the body outside the host, although occasionally, the amputation does not take place and the posterior portion is carried in (fig. 7). Although the breaking at the node may usually be caused by the activity of the host, this is not necessarily true. In one experiment a 1-day-old nymph of *Melanoplus femur-rubrum* was pinioned on a slide and 10 active preparasitic larvae were placed around it. Except for some movement immediately after it was pinioned, the grasshopper remained absolutely quiet. In about 5 minutes 2 detached post-

terior ends and 1 detached anterior end were seen; a few minutes later another detached anterior end was noticed, and finally a larva was seen actually breaking at the node. The breaking of this larva before the anterior end had penetrated the host could not have been due to any movement of the nymph.

By counting the larvae and the detached anterior and posterior ends that remain on the slide after the nymph is removed, it is possible to approximate with considerable accuracy the number of larvae that have entered. In 88 experiments in infesting first-instar nymphs of *Melanophus femur-rubrum*, 519 larvae were used, of which 304, or 58 percent, succeeded in entering. These figures were secured by actual dissection of the hosts.

#### TWO TYPES OF LARVAE

A difference in the size of eggs deposited by females of different lengths has already been mentioned (p. 163). There is an even more pronounced difference in the size of the larvae that develop from these eggs. Parasitic larvae from eggs of the smaller type range from 2 to 2.18 mm in length, the distance from the head to the node being  $370\mu$  to  $410\mu$ , and the distance from the node to the caudal extremity 1.64 to 1.77 mm. Parasitic larvae from eggs of the larger type range from 4.42 to 5.57 mm in length, the distance from the head to the node being  $610\mu$  to  $840\mu$  and the distance from the node to the caudal extremity 3.84 to 4.81 mm.

Cobb, who studied these larvae in 1923, regarded the smaller type as probably a different species. The following is quoted from unpublished notes by this investigator:

These larvae closely resemble those of *Agamermis decaudata* but with the following differences: They are much smaller; the node is differently constructed; there are about half as many trophocytes<sup>5</sup> (33); the tail end is hamate and there is a more decided difference in the diameters of the cephalic and caudal parts. In *A. decaudata* about 8 to 10 cells take part in building the node, while here apparently there is only one such cell, so that instead of presenting a roll-of-coin-like series of cells just in front of the node there is but a single, flattish, refractive cell (?) at the node. There is a difference also in the nodal contour below the subcuticle. The constriction is larger and more conspicuous than in *decaudata*, in which sometimes there is very little evidence of a constriction. Opposite the constriction in the present species is a single roll-of-coin-like cell; behind the constriction what may be called the protoplasmic portion of the interior of the nema expands, approaches closely to the cuticle, and then retreats, indicating the presence of a rather sharp encircling ridge under the cuticle immediately behind the constriction. Behind the ridge is an almost imperceptible, very shallow, second constriction. Notwithstanding these changes in the contour of the interior portion of the nema, the contour of the body itself varies but very little, there being only a decrease in diameter, sometimes almost imperceptible, opposite the before-mentioned ridge. Another marked difference in these larvae is the hooked condition of the tail end, which has somewhat the form of a fishhook, the curvature being all of  $180^\circ$ . The terminus is acute. Almost at the terminus on the ventral side there is a slight irregularity in the ventral contour, so that there is a slight asymmetry at the apex.

It should be mentioned that the number of elements (cells?) which make up the node is subject to some variation in both types of larvae, and that sometimes larvae of the larger type have the hooked caudal extremity nearly as pronounced as those of the smaller type. Except for size, the two types of larvae are certainly very similar morphologically, a condition which is equally marked in the adults.

<sup>5</sup> The term "trophocytes", as used by Cobb in this connection, refers to the cells or elements in the post-nodal part of the body and not to structures associated with the modified intestine or trophosome.

On January 4, 1928, four nymphs of *Melanoplus femur-rubrum* were exposed to active preparasitic larvae of the smaller type. An attempt was made to infest the nymphs with one parasite each. The grasshoppers were dissected on February 16. Two were not parasitized, one harbored a female mermithid 16.5 cm long, and one a female mermithid 35 cm long. This experiment should, of course, be repeated and the mermithids reared to maturity. However, several hundred experimental females have been kept under observation by the writer, and eggs of the smaller type have never been deposited by females exceeding 15 cm in length. The rearing of a female 35 cm long from a preparasitic larva of the smaller type does not support the contention that these smaller larvae are a different species. The writer is inclined to regard the size differences of eggs and preparasitic larvae as due to some host influence. It is one of the many unsolved problems that await future investigators of this group of parasites.

### PARASITIC LARVAL STAGE

The rate of parasitic larval development is subject to considerable variation. This is due in part to individual differences in the larvae, for parasites that enter the same host at the same time do not develop at the same rate. The age and physical condition of the host are also important factors. When newly-hatched grasshopper nymphs are infested the rate of development of the parasites is somewhat slower than when the nymphs are several days old. If infested nymphs are subjected to a temperature low enough to render them inactive and reduce food consumption the development of the parasites is noticeably retarded.

#### THREE DAYS IN THE HOST

There is little change in the length of the larva during the first 3 days in the host. The width increases slightly. The subdorsal esophageal gland is less conspicuous, although remnants of it can usually be seen. In one specimen, examined alive, the outline of the entire gland was visible. Although appearing nearly empty it still contained scattered masses of what seemed to be partly degenerated globules which moved backward and forward with the movement of the animal. Traces of the subventral glands may sometimes be seen, but in most cases they have disappeared. Conspicuous unicellular structures occur in the esophageal region of many mermithids and have been termed "stichocytes" by Steiner (20). They may occur as isolated structures or, as in the present case, they may lie close together and form a distinct body which has been called a "stichosome." Recent investigations by Chitwood (1) have demonstrated that each stichocyte is an esophageal gland and communicates with the lumen of the esophagus through a pore. After *Agamermis decaudata* has been 3 days in the host the stichosome contains 16 large and conspicuous nuclei, but the contour of the individual stichocytes is obscure.

#### FOUR DAYS IN THE HOST

The length of a 4-day parasite (fig. 8) is still not appreciably greater than that of a preparasitic larva from the anterior end to the node. Its maximum diameter has increased and is now 25 $\mu$  to 40 $\mu$ . The esophageal bulb has increased somewhat in length and its anterior part is drawn out into a long neck. It still undergoes rhythmic mus-

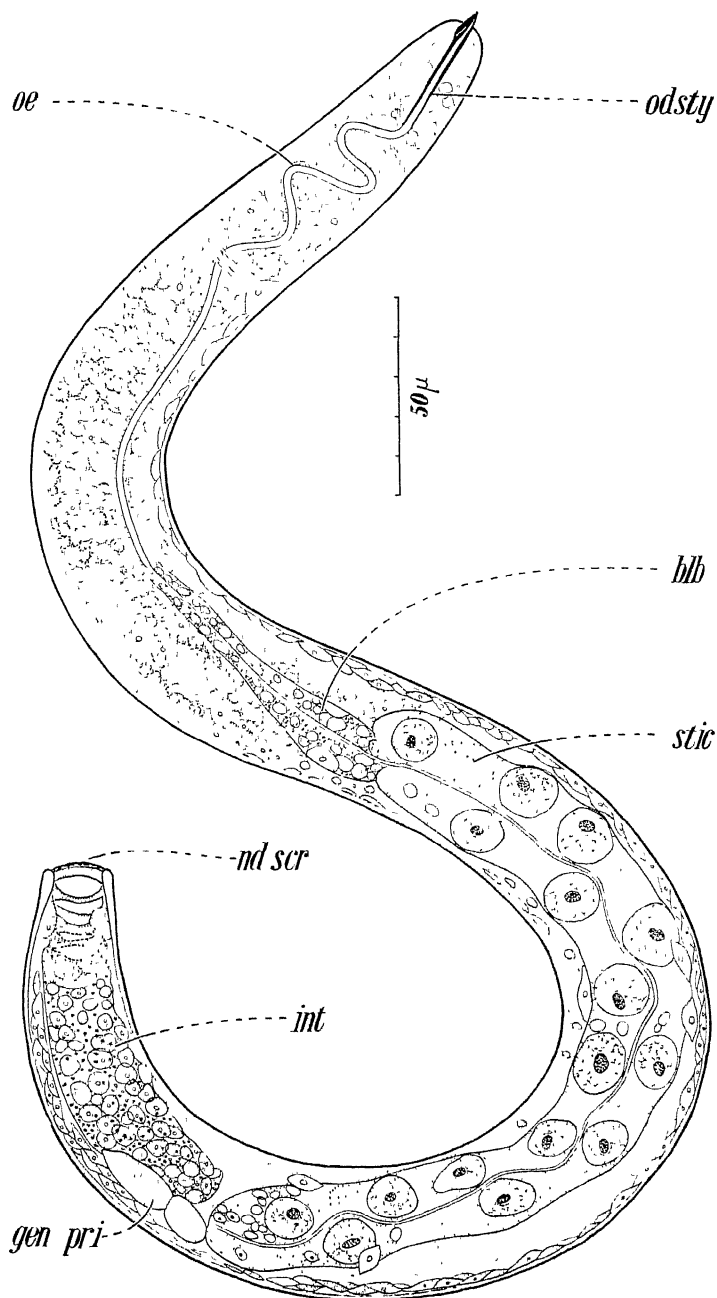


FIGURE 8.—Parasitic larva of *Agamermis decaudata* 4 days in the host *blb*, Bulblike enlargement of esophagus; *gen pri*, genital primordium; *int*, intestine; *nd scr*, nodal scar; *odsty*, odontostyl; *oe*, esophagus; *stic*, stichocytes (esophageal glands)



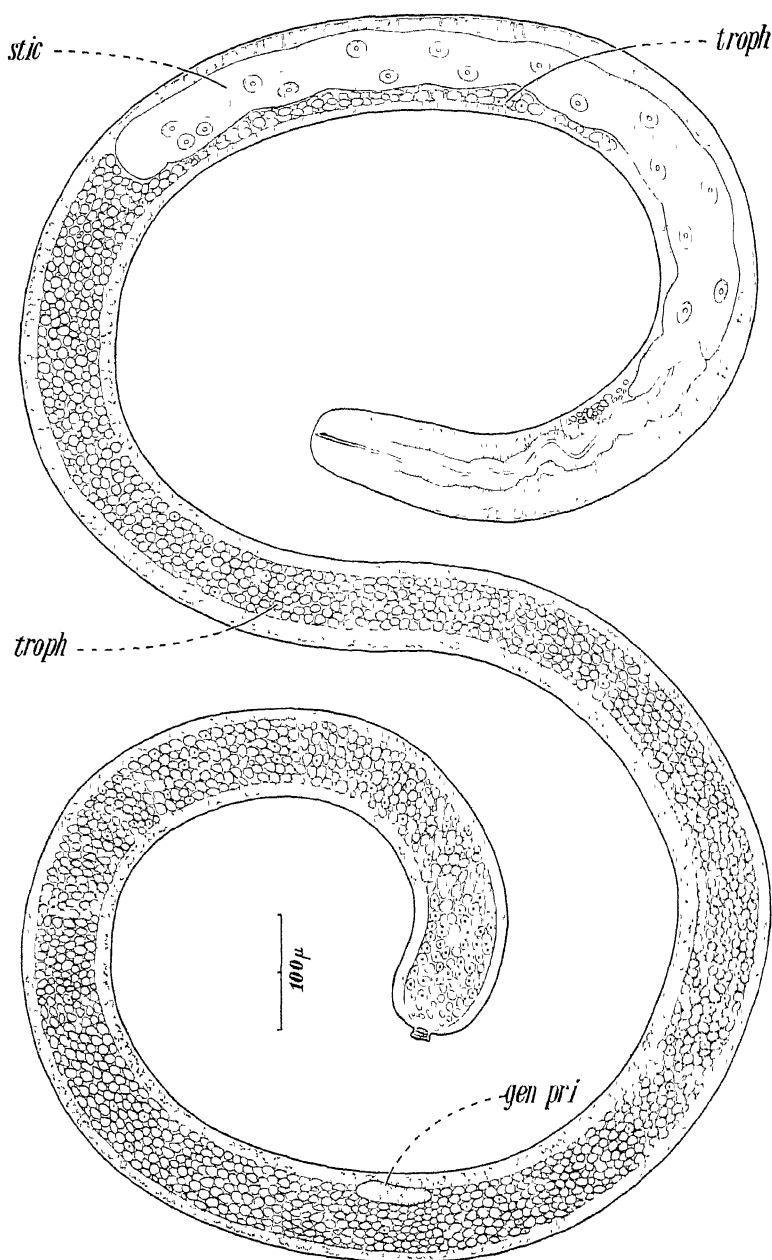


FIGURE 10.—Parasitic larva of *Agamermis decaudata* 8 days in the host: *gen pri*, genital primordium; *stic*, stichosome; *troph*, trophosome.

the year from grasshoppers collected in a particular locality show a tendency to be in approximately the same stage of development. This is in marked contrast to the case of *Mermis subnigrescens*, where it is common for a host to harbor parasites in widely different stages of development.

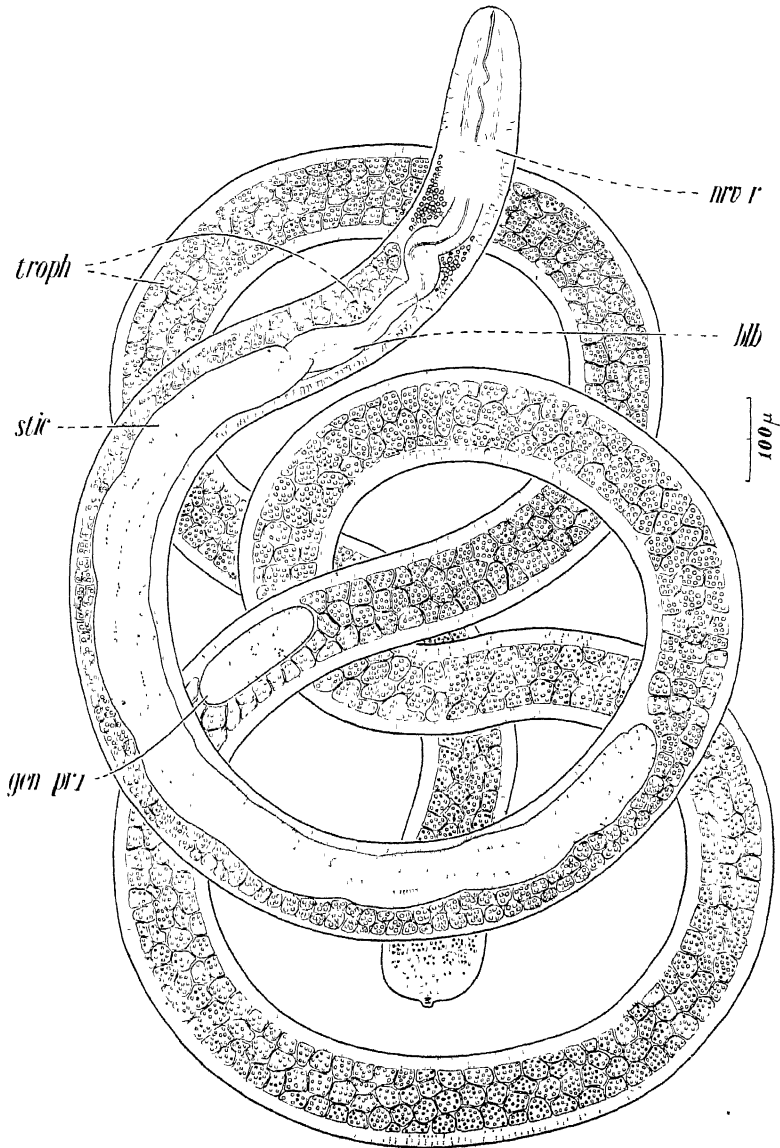


FIGURE 11.—Parasitic larva of *Agamermis decaudata* 14 days in the host *blb*, Bulblike enlargement of esophagus; *gen pri*, genital primordium, *nrv r*, nerve ring; *stic*, stichosome (esophageal glands), *troph*, trophosome.

When grasshoppers were collected in early August and confined in cages, the parasites that emerged during the following week included many males. When the grasshoppers were collected during the latter part of August, the parasites were largely females, and when the grasshoppers were collected during September, the parasites were almost exclusively females. Table 2 is based on nymphs of *Melanoplus femur-rubrum* that were reared in the laboratory and experimentally infested.

In cases where both males and females are harbored by the same host, all frequently emerge simultaneously at about the normal time for the emergence of the males. Some of these prematurely emerged females survive and some die, depending, apparently, on the extent of development at the time of emergence.

While the duration of the parasitic stage is subject to considerable variation, it is usually from 1 to 1½ months for males and from 2 to 3 months for females.

TABLE 2.—Duration of the parasitic larval stage of *Agameris decaudata*

Parasites per host				Period from infestation to emergence	Parasites per host				Period from infestation to emergence
Females	Males	Sex not determined	Total		Females	Males	Sex not determined	Total	
Number	Number	Number	Number	Days	Number	Number	Number	Number	Days
-----	2	2	4	30	1	-----	-----	1	81
-----	2	-----	2	33	1	-----	-----	1	81
2	2	-----	4	38	-----	2	-----	2	35
-----	2	-----	2	44	-----	5	1	6	31
-----	2	-----	2	31	-----	-----	-----	-----	-----

#### EFFECTS OF THE PARASITE ON THE HOST

There is no doubt that grasshopper nymphs can be infested with a sufficient number of *Agameris decaudata* to cause the death of the host. It is difficult to determine how many are necessary. The writer at one time regarded from 8 to 10 as usually constituting a lethal infestation, but this estimate was based on hosts reared in the laboratory and may not be true for grasshoppers in their natural environment. The number of parasites that an animal will sometimes tolerate is one of the astonishing things about parasitism.

Although over 3,000 infested grasshoppers, collected in the vicinity of Falls Church, Va., have been examined, only 1 harbored as many as 4 *Agameris decaudata* parasites, and instances of 2 and 3 parasites per host were comparatively rare. But, strangely enough, on one occasion 7 larvae of this species were dissected from the small coccinellid beetle *Ceratomegilla fuscilabris* Mulsant. That grasshoppers can be infested with a large number of parasites of this species has been demonstrated in the laboratory, where as many as 27 larvae have entered 1 nymph. However, one parasite per host (fig. 12) appears to be the usual number in nature, and evidently death from overinfestation is not a common occurrence.

Mermitids usually kill their host at the time they emerge. Crawley and Baylis (6), in the case of ants, found that "after the emergence of the worm the ants became very active but their death was only

deferred a short time." Strickland (21) states that *Simulium* larvae were always finally killed when the mermithid bored its way out to escape into the water. Glaser and Wilcox (8, p. 13) observed, in the case of *Melanoplus atlantis* and *M. bivittatus*, that "when the worms are about to emerge, the grasshoppers fall over on one side, kick for a time, and then die."

The writer has observed the emergence of *Agamermis decaudata* many times. The parasites came out head foremost, forcing their way through the body wall between the segments, most often in the region of the thorax. In no case were they seen emerging through the mouth or anus. A half hour or more was often required for the process, during which time the grasshopper might be quite active, dragging the mermithid around and otherwise treating it roughly, a fact which may account for the rather common occurrence in nature of injured postparasitic larvae and adults. In some cases the nema, when about half way out, coiled around a blade of grass and entirely extricated itself as the grasshopper moved away.

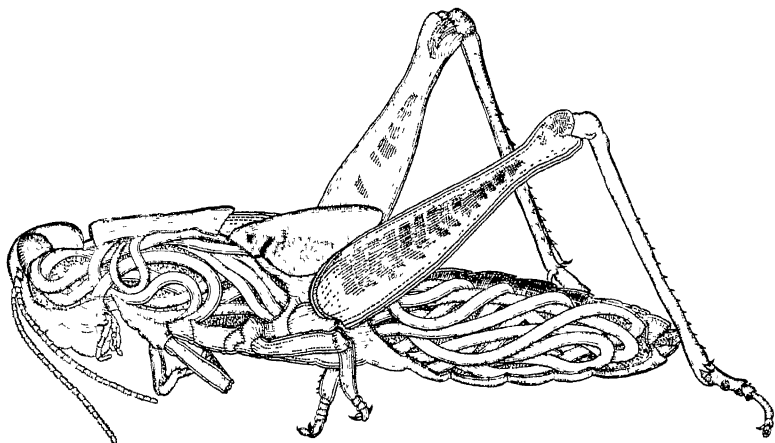


FIGURE 12.—Nymph of *Melanoplus femur-rubrum* with parts of body wall dissected away to show position of parasite. This grasshopper contains one female *Agamermis decaudata*, which is approximately full-grown.

The immediate effect upon the grasshopper is a partial paralysis of the legs and an inability of the insect to maintain its equilibrium. It will fall over on one side, then right itself and crawl slowly around, using its fore and middle legs with apparent difficulty and often dragging its hind legs. These movements become more sluggish until finally it falls over and remains quiet as if dead, but will move if disturbed, and may live for several hours. A grasshopper from which a mermithid has emerged during the day may still be alive when the laboratory is closed for the night, but it is always dead the following morning.

The external structure of some insects is noticeably changed by the presence of mermithid parasites. This is especially true in the case of ants. Inasmuch as Wheeler (22) has carefully discussed this phase of the subject, it will not be reviewed here. The writer has not been able to detect, at any stage in their development, a noticeable external difference in structure between parasitized and nonparasitized grasshoppers.

In every case where the writer has examined an adult female *Melanoplus femur-rubrum* parasitized with *Agamermis decaudata*, the ovaries were reduced in size (fig. 13), and in many instances were vestigial. The extent to which the female gonads are reduced depends on the time the infestation was acquired and the number and size of the parasites. In the case of *Mermis subnigrescens*, where infestation may take place at any stage in the life of the host, the time of infestation and the number of parasites become important factors in this connection. With *A. decaudata*, of which one parasite each is usually acquired while the nymphs are young, these factors cause less variation in the female gonad development of their hosts. When an adult grasshopper is found parasitized with a single *A.*

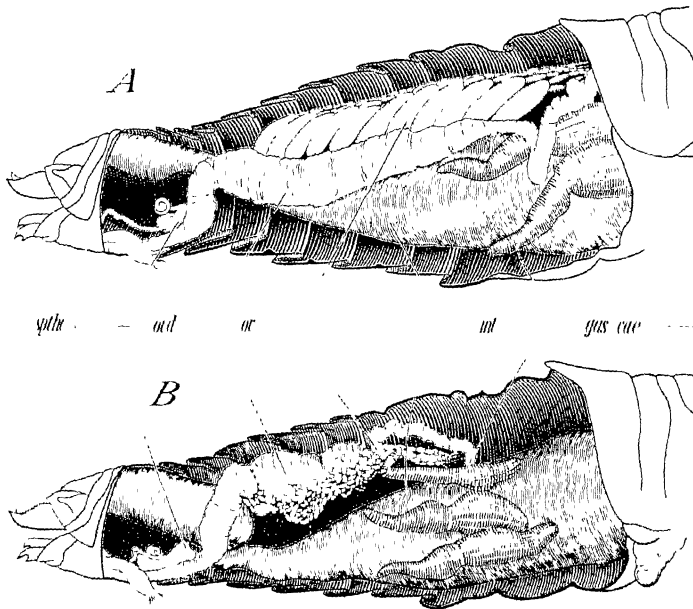


FIGURE 13 — *Melanoplus femur-rubrum* dissected to show female reproductive organs of (A) normal adult and (B) one from which *Agamermis decaudata* has emerged. Gas cae, gastric caeca; int, intestine; or, oval; ord, oviduct; spha, spermatheca.

*decaudata* the parasite is usually a female. This is probably because males of this species, having a shorter parasitic stage, usually emerge before their hosts reach maturity. Consequently the cases of female gonad suppression that have come to the writer's attention have been those in which grasshoppers harbored relatively large mermithids. To what extent the smaller male parasites affect the female gonad development of their hosts is not known.

The male gonads of *Melanoplus femur-rubrum* are much less affected by the presence of mermithids than are the female gonads. In many instances the testes are not materially reduced in size, and all parasitized male grasshoppers examined by the writer seemed capable of producing spermatozoa. In several instances copulating pairs of both

*M. femur-rubrum* and *M. bivittatus* have been captured, and on examination the male was found to harbor a mermithid.

Specimens of *Melanoplus femur-rubrum* collected from the middle to the latter part of September were largely adults, with a few individuals still in the nymphal stage. In several instances nearly all such nymphs were parasitized, although not over 10 percent of the adults were infested. The probable explanation of this became apparent when it was noted that in cage-reared grasshoppers parasitized individuals were still in the nymph stage long after uninfested individuals, hatched at the same time, had reached maturity. This retarded development of infested grasshoppers is sometimes very pronounced.

#### EFFECTS OF THE HOST ON THE PARASITE

In an earlier paper (2) it was demonstrated that in the case of *Mermis subnigrescens* a large number of mermithids per host resulted in the development of male parasites and a small number of mermithids per host in the development of females. It was suggested that this is probably also true in the case of *Agameremis decaudata*. The manner in which *M. subnigrescens* is acquired often results in successive infestations, and a single host may harbor 20, 30, or, on rare occasions, over 100 parasites. In the case of *A. decaudata*, as already noted, a grasshopper harboring more than two or three parasites has rarely been found. Nevertheless, males of *M. subnigrescens*, while easily reared in the laboratory, are seldom encountered in nature, few specimens ever having been collected. Males of *A. decaudata*, on the other hand, are always more numerous than the females and are easily collected in large numbers. Males of this species can and do develop in grasshopper hosts, although when these insects are collected and confined in cages the number of males that emerge is surprisingly small. But insects other than grasshoppers are infested with *A. decaudata*. In the vicinity of Falls Church, Va., the coccinellid beetle *Ceratomegilla fuscilabris* and the leaf hopper *Draeculacephala mollipes* (Say) both regularly harbor this parasite, and on several occasions unidentified crickets have been found infested. When the coccinellid beetles were collected and confined in cages, the few parasites that emerged were males. It seems probable, therefore, that some of these smaller insect hosts contribute toward the development of males of *A. decaudata*.

#### COMPARATIVE INFESTATION OF MALE AND FEMALE GRASSHOPPERS

When examining *Melanoplus bivittatus* and *M. atlantis* collected near Dummerston Station, Vt., Glaser and Wilcox (8) found parasitized, on an average, about 45 percent of the females but only about 9 percent of the males. The data in table 3, based on an examination of over 3,000 specimens composed largely, but not exclusively, of three species, namely, *M. femur-rubrum*, *Orchelimum vulgare*, and *Conocephalus brevipenne* (Scudder), show no such difference between the percentage of infestation in the two sexes.

Table 3 was compiled from dissections begun in early July and continued throughout the year as long as grasshoppers could be collected. A large percentage of the material examined, however, consisted of nymphs.

## POSTPARASITIC LARVAL STAGE

## MIGRATION TO THE SOIL

Once free from the host, the postparasitic larvae enter the soil. This migration is probably one of the vulnerable points in the life cycle. In breeding cages where the soil is hard or crusted over, many newly emerged parasites perish, and one often finds their dried and tightly coiled remains on the surface of the soil or clinging to foliage put in to serve as food for the grasshoppers. It seemed possible that wet or humid conditions might stimulate fully grown parasitic larvae to emerge, and the writer attempted to test this by thoroughly wetting cages containing infested grasshoppers, but was unable to show in a convincing manner that the water had any effect. In every case where emergence has been observed it took place during the day, showing that this act is at least not confined to the night. In nature, the soil ordinarily is not very hard or dry beneath thick herbaceous vegetation and the ground is penetrated to a surprising extent by the galleries of various organisms.

TABLE 3—Comparison of the number of male and female grasshoppers infested with *Agameris decaudata*

Family	Males			Females		
	Total examined	Infested		Total examined	Infested	
	Number	Number	Percent	Number	Number	Percent
Acrididae <sup>1</sup> .....	981	105	10.7	1,304	134	10.2
Tettigoniidae <sup>2</sup> .....	367	72	19.6	457	67	14.6

<sup>1</sup> Largely *Melanoplus femur-rubrum*.<sup>2</sup> Largely *Orchelimum vulgare* and *Conocephalus brevipes*.

Females usually penetrate the soil to a depth of from 5 to 15 cm, where they coil themselves into the characteristic knot (fig. 14). Here they molt, copulate, deposit their eggs, and die. The vertical distribution of these mermithids beneath an area of 9 square feet in a field at Falls Church, Va., is shown in table 4. Both adults and postparasitic larvae are included.

TABLE 4.—Vertical distribution of *Agameris decaudata* in the soil

Depth (cm)	Males	Females	Total	Depth (cm)	Males	Females	Total
	Number	Number	Number		Number	Number	Number
1 to 5.....	1	2	3	20 to 25.....	1	1	2
5 to 10.....	37	24	61	25 to 30.....	0	0	0
10 to 15.....	12	6	18				
15 to 20.....	4	1	5	Total.....	55	34	89

Males apparently do not seek the females until the latter have molted or are about to molt, and during the first winter individuals of the two sexes remain isolated. The knot of the postparasitic larval female is composed of loose loops, in contrast to the tight knot usually formed by adults.

## DURATION OF THE POSTPARASITIC LARVAL STAGE

Like other stages in the development of this parasite, the duration of the postparasitic larval stage varies somewhat with different indi-

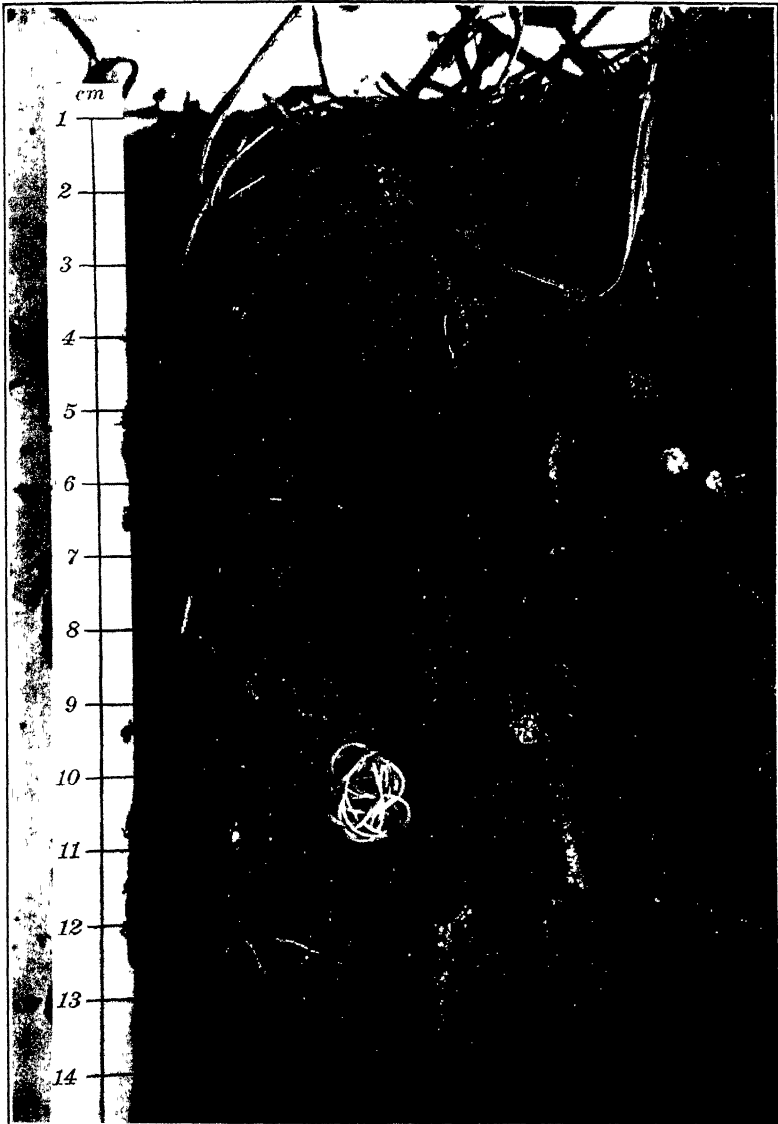


FIGURE 14 —A knot of *Agamermis decaudata*, containing a female and several males, in natural position in the soil.

viduals. A majority of the females molt during July and begin depositing eggs in August. Information regarding males is less satisfac-



tory, but their postparasitic larval development appears to parallel that of the females (table 5).

TABLE 5—Duration of the postparasitic larval stage of *Agamermis decaudata*

Larva no	First examination June 8, about 9 months after emergence	Second examination July 11, about 10 months after emergence	Third examination August 6, about 11 months after emergence
1.....	Molt starting, specimen injured and preserved	Outer cuticle separating at anterior and posterior ends	Cuticle not entirely off
2.....	No sign of an approaching molt		
3.....	Molt fairly well under way. Nema in poor condition. Preserved.		
4.....	Cuticle separating at extremities of body	Lost.	Completely molted. Eggs not seen
5.....	No sign of approaching molt	Outer cuticle separated at anterior end	
6.....	.....do.....	Not examined.....	
7.....	.....do.....	Completely molted	Completely molted. Had already deposited eggs. Oldest embryos 6 to 8 days old.
8.....	.....do.....	Outer cuticle separated at extremities of body	
9.....	.....do.....	Not examined.....	
10.....	.....do.....	Molt complete.....	Completely molted, deposited numerous eggs. Oldest embryos 8 to 10 days old.
			Lost

The duration of the postparasitic larval stage of females is about 9 to 11 months and, roughly, extends from September to July. This appears to be true for males also, although the time of their molting has not been carefully determined.

#### THE NODAL SCAR

The elements that constitute the node of the preparasitic larva form a plug to prevent the body contents from exuding after the caudal portion of the body is broken off. This structure persists as the nodal scar throughout larval development. It is a conspicuous anatomical feature while the larva is small but becomes increasingly less conspicuous as the larva grows, and is difficult to see in the postparasitic larval stage. Its presence constitutes convincing evidence that no molt takes place during parasitic development.

Immediately before the final molt, females have a yellowish or brownish tinge. The old cuticle first separates at the extremities of the body, then breaks transversely a short distance posterior to the head, and the anterior and posterior portions come off over the head and tail respectively. In males, the yellowish tinge preceding the final molt is not so noticeable but the cuticle is shed in a similar manner and at approximately the same time as is that of the females.

#### ADULT STAGE

At the time of the final molt both males and females are very opaque. This opaqueness is due primarily to the trophosome or fat body (modified intestine), which fills all available space in the body. This structure probably contains the reserve material for all future metabolism. As this reserve material is gradually consumed the body of the mermithid becomes less and less opaque, and when it is exhausted the animal dies.

In the case of these recently molted first-year females, oviposition begins nearly a year after emergence from the host, sometimes in July but usually during August, and continues until interrupted by cold weather. It is resumed the following June.

Three gravid females were dug from the soil on November 27, and washed free of eggs. Each female, together with the accompanying males, was placed in a cavity between two disks of soil, and each pair of soil disks was placed in a tin box. The boxes were buried 15 cm below the surface of the soil. From February until July these specimens were examined at intervals of about 2 weeks. Eggs were first seen on July 12, although it was believed that in all three cases egg laying had begun 10 to 12 days earlier.

The foregoing experiment, corroborated by field observations, indicates that second-year females begin depositing eggs somewhat earlier in the summer than do recently molted first-year females. At the end of the second summer in the soil, females are very transparent and the trophosome is nearly exhausted. Experimental animals usually failed to survive a third winter; two that were recovered alive the following spring were in a nearly exhausted condition and seemed incapable of depositing more eggs.

A female collected during the winter is surrounded by all the eggs deposited during the preceding summer. Egg counts were made on six females by the following method. Each knot, together with the surrounding soil, was placed in a graduated cylinder, and water was added to make 100 cc. After a thorough shaking, a 5-cc sample was removed; the eggs were counted and the total number computed. The totals were 2,625, 6,530, 5,420, 5,680, 4,780, and 3,430 respectively. As eggs are deposited during two summers, these values may represent about half the total output.

In a former paper (2) it was pointed out that when females are not permitted to copulate they do not deposit eggs. Virgin females, after remaining in the soil for as long as 14 months, oviposit when males are provided. In view of the prevalence of males, it seems unlikely that in nature females are often rendered unproductive through lack of mates. Each female is usually accompanied by from 2 to 4 males, and in one instance a knot was found composed of a female and 24 males. Occasionally 2 females will be found in the same knot, but such instances are rare.

As previously noted, second-year females do not usually resume egg laying until June. Females kept in the laboratory at room temperature deposit eggs at any time throughout the year. The suppression of egg laying during the winter seems, at first glance, to be a matter of temperature. However, moisture is probably also an important factor. If the soil in which ovipositing females are kept is moistened beyond a certain point egg laying stops. In experimental work considerable care must be exercised in regulating moisture if normal egg laying is to be maintained. In the vicinity of Falls Church, Va., *Agamermis decaudata* is confined to the so-called "bottom" lands. The soil in these localities is usually saturated with water throughout the winter and is frequently flooded for short periods. By June the soil usually has become sufficiently dry to be tillable. Under these conditions it seems probable that moisture is an important factor in retarding egg laying during early summer.

## SUMMARY OF LIFE CYCLE

The following summary of the life cycle of *Agamerms decaudata* is based on observations made at Falls Church, Va. The grasshoppers referred to are *Conocephalus brevipenne* and *Melanoplus femur-rubrum*, two of the commonly infested species in this locality. The different changes are indicated as taking place during certain periods at various times of the year. Although the outline as given is essentially correct, occasionally an individual may undergo a certain change before or after the time indicated. A few preparasitic larvae enter hosts at least as early as April or as late as August, and the time of emergence is correspondingly affected. All males do not necessarily emerge during July nor all females during August. It is not known to what extent the life cycle may be changed when insects other than grasshoppers serve as hosts.

*August.*—Knot *a* (fig. 15) is composed of one female *Agamerms decaudata* and several males that emerged from their hosts 2 years previously. The female began depositing eggs during August of the preceding year. Knot *b* is composed of one female and several males that emerged from their hosts 1 year previously and molted the preceding July. The female is now beginning to deposit eggs for the first time. Parasites of the present season, which would now be emerging, are omitted for the sake of brevity. After these have emerged and before those in knot *a* have died there exist simultaneously in the soil mermithids of three ages. Grasshoppers are also depositing eggs, *Melanoplus femur-rubrum* in the soil and *Conocephalus brevipenne* in the stems of plants.

*September and October.*—The female mermithid in knot *a* and the one in knot *b* (fig. 15) continue to deposit eggs, and by October several thousands have accumulated around each.

*November and December.*—The females of knots *a* and *b* (fig. 16) stop laying eggs with the advent of cold weather. The mermithids in knot *a* have nearly exhausted their supply of stored food material and will not survive the winter.

*January and February.*—An occasional egg from knots *a* and *b* (fig. 16) may hatch.

*March and April.*—A few eggs from knots *a* and *b* (fig. 16) continue to hatch, the number increasing slightly.

*May.*—Grasshopper eggs begin to hatch during the latter part of the month. There is an increase in the number of *Agamerms decaudata* eggs which are hatching, and the infestation of nymphs begins.

*June.*—The remaining eggs in knots *a* and *b* (fig. 17), which comprise a very large percentage of those present the preceding autumn, now hatch. The larvae migrate to the surface of the soil, then seek out and enter the young grasshopper nymphs, probably climbing the vegetation while it is wet with dew or rain.

Eggs are hatching which have been deposited by females of two ages, namely, the female *a*, which emerged about 31 months previous and is now dead, and the female *b*, which emerged about 19 months previous and which will continue to deposit eggs throughout the summer.

*July.*—The eggs in knots *a* and *b* (fig. 17) have all hatched and the period of infestation is over except for an occasional belated infestation. The female in knot *b* now resumes egg laying, not having deposited eggs since the previous November.

*August.*—Uninfested grasshoppers are now depositing eggs. Larval male mermithids are emerging from their hosts and entering the soil.

*September.*—Larval female mermithids are emerging from their hosts and entering the soil.

*October.*—The female in knot *b* (fig. 18) continues to deposit eggs. Newly emerged males (*c*, ♂) and females (*c*, ♀) form separate knots in the soil.

*November and December.*—The female in knot *b* (fig. 19) stops laying eggs; she is now surrounded by several thousand eggs.

*January and February.*—An occasional egg from knot *b* (fig. 19) may hatch.

*March and April.*—Eggs from knot *b* (fig. 19) continue to hatch, the number increasing slightly.

*May.*—Grasshoppers now begin to hatch. The eggs from knot *b* (fig. 20) hatch in increasing numbers and the infestation of nymphs begins. The mermithids in knot *b* are dead.

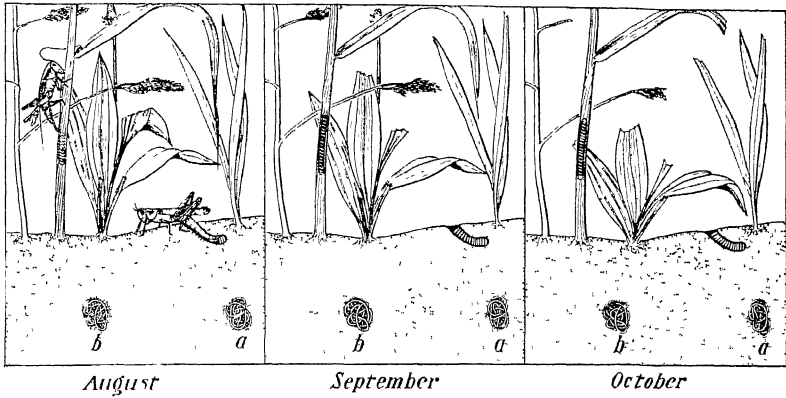


FIGURE 15.—Life cycle of *Agameremis decaudata*, August to October. *a*, Knot composed of one female and several males that emerged from their hosts 2 years previously; *b*, knot composed of one female and several males that emerged from their hosts 1 year previously.

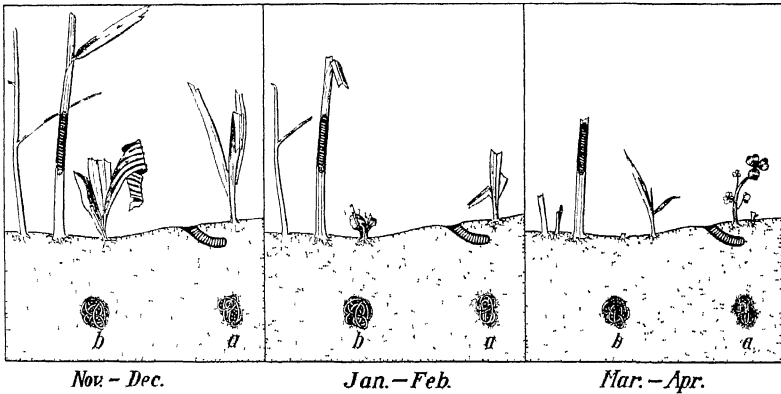


FIGURE 16.—Life cycle of *Agameremis decaudata*, November to April. *a* and *b*, Knots as described in figure 15.

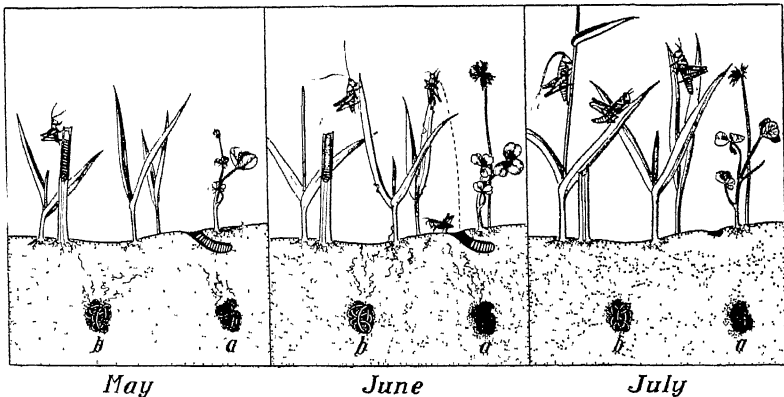


FIGURE 17.—Life cycle of *Agameremis decaudata*, May to July. Larvae issuing from knots at *a* and *b*; female in knot *a* now dead; female in knot *b* resumes egg laying.

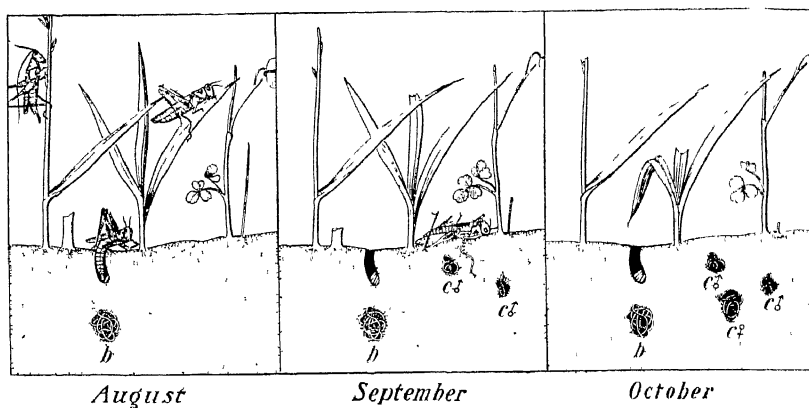


FIGURE 18.—Life cycle of *Agameris decaudata*, August to October. Female in knot *b*, continues to deposit eggs; males (*c*, ♂) and females (*c*, ♀) emerge from grasshoppers and form separate knots.

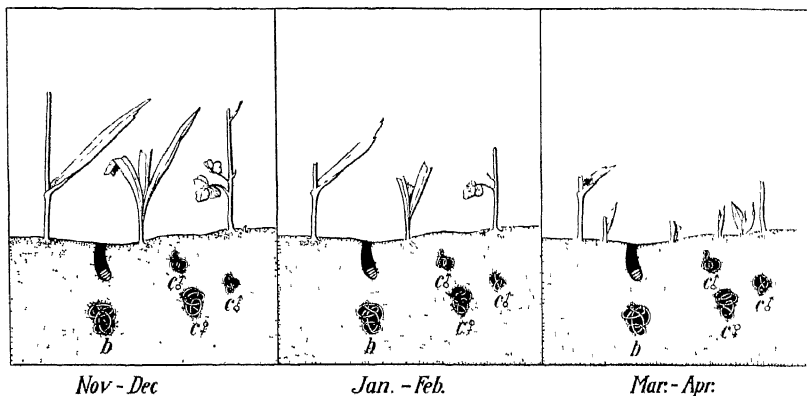


FIGURE 19.—Life cycle of *Agameris decaudata*, November to April. *b*, Knot containing female and seven thousand eggs; *c*, ♂, and *c*, ♀, knots containing male and female larvae, respectively.

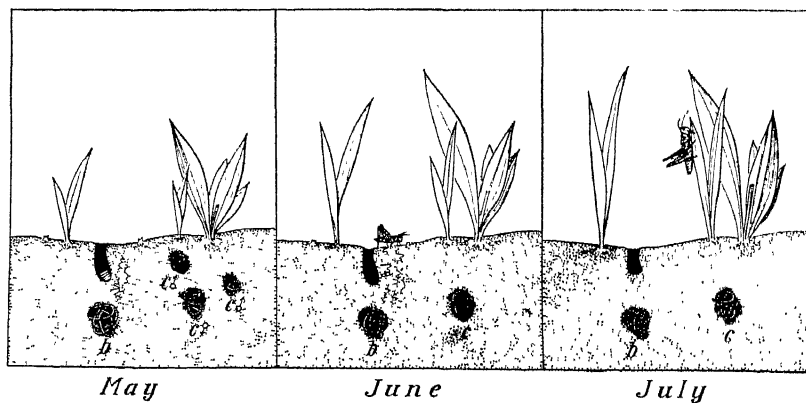


FIGURE 20.—Life cycle of *Agameris decaudata*, May to July. Adults in knot *b* dead, larvae issued from knot *b*, males from knot *c*, ♂, join females in knot *c*, ♀, forming knot *c*.

*June*—The remaining eggs from knot *b* (fig. 20) now hatch, and the infestation of grasshopper nymphs takes place as during the previous June. The more recently emerged postparasitic larvae (c, ♂, and c, ♀) are preparing to molt and males have joined or are about to join the females.

*July*.—The female and males in knot *c* (fig. 20) are now molting and the female will begin to deposit eggs during August.

#### SUMMARY

Mermithids are common nematode parasites of many insects. *Agamermis decaudata*, a parasite of grasshoppers and some other insects, is found throughout the northeastern part of the United States, southward to Virginia and westward to Nebraska. The present paper is based on studies made at Falls Church, Va.

The adults of this parasite occur in the soil, where eggs are deposited. Hatching takes place between late June and mid-July. Larvae migrate to the surface of the soil, climb the vegetation, and enter the body cavity of recently hatched grasshopper nymphs by penetrating the body wall. The parasites remain in the host from 1 to 3 months and complete their growth. They then emerge by forcing their way through the body wall and enter the soil. Molting takes place the following summer and females begin depositing eggs.

Infested grasshoppers are retarded in their development and females are usually rendered sterile. The emergence of the parasite causes the death of the grasshopper. The morphological development of the parasite is discussed.

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# CHROMOSOME DIFFERENCES IN A WHEAT-RYE AMPHIDIPLOID<sup>1</sup>

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## INTRODUCTION

Plant hybrids that contain the diploid chromosome complement of the parents have been described in recent years by a number of investigators. They occur rarely and are produced in combinations which usually are highly infertile or self-sterile in the  $F_1$  generation. Some investigators have designated such hybrids as "amphidiploids" to call attention to the presence in one plant of both parental chromosome sets in the diploid condition. Others, principally the Russian workers, have called them "balanced hybrids" to emphasize the paired or balanced condition of the chromosome elements.

Most of the known amphidiploids have been produced from interspecific and intergeneric artificial hybrids. They are of special interest to geneticists and cytologists on account of the light they shed on methods of species formation and also on account of their possibilities for plant improvement.

## LITERATURE REVIEW

Meister (3)<sup>3</sup> first reported in 1927 the discovery by his associates and himself of a 56-chromosome wheat-rye amphidiploid.

Tjumjakoff (9) mentioned the probable polyploid nature of the hybrids, and Meister (4, fig. 6) showed a spike of this unusual type of wheat-rye hybrid, stating that it was apparently of tetraploid make-up.

Meister (5) gave a botanical description of the Saratov wheat-rye amphidiploids and designated the new species combination as *Triticum secalotriticum saratoviense* Meister.

The polyploid nature of these constant, fertile or partially fertile hybrids was determined by the cytological investigations of Levitsky and Benetzkaia (2). They found in the somatic cells of the hybrid 56 chromosomes and in the gametic cells 28 chromosomes. The somatic number given was established for nine individuals of the  $F_4$ ,  $F_5$ , and  $F_6$  generations in three different families.

In this material they observed both normal and irregular chromosome behavior in the meiotic divisions. In some division figures from two to six or more lagging univalents were found. The univalents afterward split longitudinally, sometimes with irregular scattering of

<sup>1</sup> Received for publication Oct. 3, 1935; issued March 1936.

<sup>2</sup> The writer wishes to express appreciation to C. W. Hungerford, chief of the Division of Plant Pathology and dean of the Graduate School, University of Idaho, for the use of his excellent research microscope and for laboratory space and equipment; also to J. W. Taylor, associate agronomist, Division of Cereal Crops and Diseases, Bureau of Plant Industry, U. S. Department of Agriculture, for supplying the hybrid material which made this work possible.

<sup>3</sup> Reference is made by number (italic) to Literature Cited, p. 204.



chromosomes over the spindle. Chromosome elimination into the plasma was observed, also complete disintegration of amorphous elements, such as microcytes, cell fragments, etc.

Judging by the number of lagging chromosomes, Levitsky and Benetzkaia estimated that probably at least 50 percent of the pollen grains contained an increased number of chromosomes (more than 28), yet all of the parental plants examined had 56. They conclude:

Thus it remains to suppose that these normal gametes, having arisen from normal divisions, leave behind with their pollen tubes all other gametes with deviating chromosome numbers \* \* \*.

The formation of amphidiploids is believed by these authors to result from "apogamous development of the ovule in  $F_1$ ."

Tjumjakoff (10), in a study of the fertility of these wheat-rye hybrids of balanced type, found in each of two lines that the average number of kernels per spikelet in normal spikes was 1.2. The following year the average number of kernels per spikelet of the  $F_4$ , when sown in pots, was 0.9. In the former this would be a fertility of about 50 to 60 percent for the common wheats under average conditions.

A brief description of the plant characters and of certain chromosome differences in an artificially produced wheat-rye amphidiploid is reported herein.

#### MATERIAL AND EXPERIMENTAL DATA

An individual plant head of a wheat-rye hybrid having the hairy-neck rye character was obtained from the Arlington Experiment Farm, near Washington, D. C., in the winter of 1930. This hybrid had been under observation for a number of years by J. W. Taylor, who made the original wheat-rye cross, the backcrosses, and the selection.

The origin of this amphidiploid was indicated by Taylor and Quisenberry (8) in a report on the inheritance of rye crossability in wheat hybrids. Purplestraw wheat was crossed with Abruzzes rye in 1923. The  $F_1$  was backcrossed to Purplestraw, producing two seeds from more than 1,000 pollinations. The progeny of one of these seeds, consisting of 10 plants, was backcrossed with rye. Only two of the 20  $F_1$  hybrid plants from backcrossing with rye dehisced and one of these produced a single selfed seed. Taylor and Quisenberry state: "From this seed originated a so-called nonsegregating line intermediate between wheat and rye" (8, p. 150).

Plants from this amphidiploid were grown in the agronomy garden of the University of Idaho for 2 years. In both years they were low in vigor at the beginning of the growing season, but before the end of the season they developed into fairly vigorous plants, which in most characters were intermediate between the parents.

The leaves and culms were faintly to definitely purple in the early stages of growth. The mature plants were mid-tall with weak to mid-strong culms and with the upper peduncles hairy. The kernels were red, mid-sized to large, mid-long to long, slightly shriveled to medium plump, and somewhat resembled rye. The fertility was low to medium, the set of seed being comparable to that of the amphidiploid reported by Meister (3).

A small sample of seed of one of the Saratov selections was obtained <sup>4</sup> for seeding at Moscow, Idaho, in 1932 and 1933. This amphidiploid was more vigorous than the one obtained from Arlington farm. At maturity the spikes contained many empty florets.

Root tips from the Arlington farm amphidiploid were obtained by the writer from plants grown in the greenhouse. These were embedded in paraffin and taken to the University of Idaho, where the

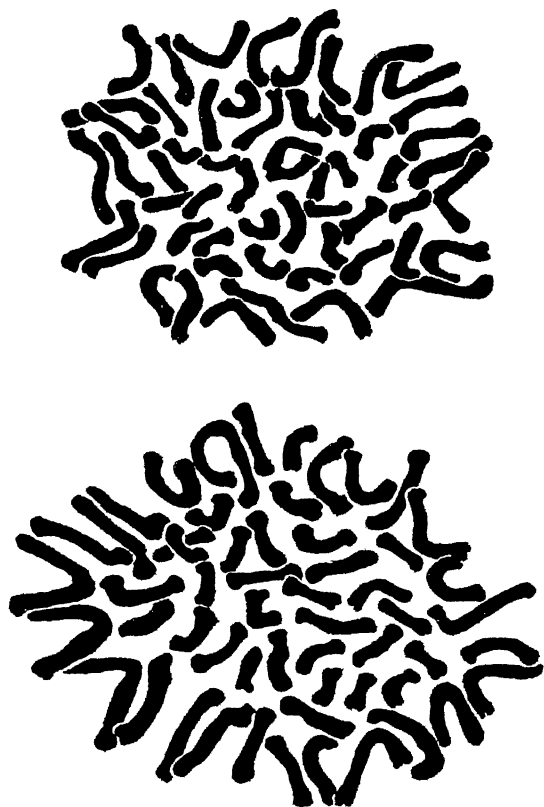


FIGURE 1.—Camera-lucida drawings showing two somatic metaphase plates of  $F_2$  Purplestraw wheat  $\times$  Abruzzes rye amphidiploid. ( $2n=56$ .)  $\times$  about 1,375.

cytological study of the material was made in 1931. S. Navashin's chrom-acetic-formalin was used as the killing and fixing agent and Heidenhain's haematoxylin as the stain.

The chromosome number of the somatic cells was found to be 56. Counts were made on 6 cells; 5 had 56 chromosomes, and 1 had 58. The general shape and comparative size of these chromosomes are shown in figure 1. It was not possible to distinguish between the wheat and the rye somatic chromosomes.

In 1933 pollen mother cells of the amphidiploid were examined by Belling's aceto-carmine method. Counts were made on a number of

<sup>4</sup> Through the courtesy of E. F. Gaines, cerealist, of the Washington Agricultural Experiment Station.

different cells at first metaphase, and all were found to contain 28 chromosome pairs. Three polar views of first metaphase plates are shown in figure 2. The chromosome number indicated thus agrees with that found in the root tips.

Certain chromosome type differences were noted in the first metaphase of some pollen mother cells, whereas none was found in the somatic cells. In countable cells a number of large bent chromosomes usually were found scattered along the edges or outside of the more or less well-defined, centrally located group of typical bivalent chromosomes. Up to seven of these large, light-staining border chromosomes were found in some cells and they probably were those of rye. It is as-

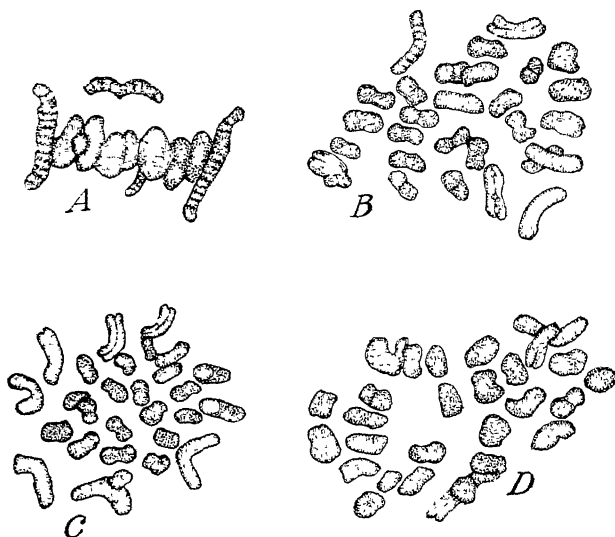


FIGURE 2.—Camera-lucida drawings of 4 meiotic-division metaphase plates of  $F_1$  Purplestraw wheat  $\times$  Abruzzes rye amphidiploid. ( $\times$  about 1,000.) A, Equatorial view of first metaphase plate showing typical arrangement of the wheat bivalents, with the large rye chromosomes (here banded) at the edges or outside of the wheat group. B, Polar view of heterotypic metaphase plate. Note the 6 large chromosomes at the borders. ( $n=21 \text{ II W}+7 \text{ II R}$ ) C, Polar view of heterotypic metaphase plate. Note the 7 large bent-border chromosomes ( $n=21 \text{ II W}+7 \text{ II R}$ ) D, Polar view of heterotypic metaphase plate. Note 3 or 4 large bent chromosomes at the plate border. ( $n=28 \text{ II.}$ )

sumed in the following discussion that the large chromosomes were those of rye and the smaller chromosomes were those of wheat. In previous work with wheat  $\times$  rye hybrids this tendency for the rye chromosomes to retreat to the edge or outside of the main group had been observed (1). This behavior apparently is due to incompatibility between the two sets of chromosomes as a result of their genetic differences. Figure 2, C, shows seven large light-staining bent-border chromosomes. The two large straight ones at the right represent wheat chromosomes, the ends of which evidently had been pressed flat by the cover glass. Figure 2, A, B, and C, shows large bent-border chromosomes in varying numbers.

The wheat bivalents were somewhat smaller than the rye bivalents, usually more or less constricted at or near the middle, sometimes with a faint longitudinal line indicating the line of union of the paired chromosomes. They also stained more deeply. Some of the rye

bivalents were shaped like those of the wheat but were larger and absorbed the stain less readily; others were large and curved, sometimes showing the heterotypic (or homeotypic) split and were also definitely lighter staining. These chromosome types are shown in figure 2, *B*, *C*, and *D*.

Some of the large lighter stained border chromosomes were banded transversely. These bands were dark red and opaque in contrast to the lighter red areas between them. One banded chromosome is shown in figure 2, *B*, and another in *C*. Other banded chromosomes were present in *C* but are not shown. Figure 2, *A*, shows an equatorial view of a metaphase plate having 4 of the large border chromosomes, all banded, 1 of which is outside the equatorial plate.

### DISCUSSION

The differences noted in staining reaction between the heterotypic chromosomes of wheat and rye may be explained partly by variable stages of development in the two types and partly by variations in composition. The differences observed among the rye chromosomes in some cells might result from variations in the rye chromosomes themselves, i. e., those possessing the greatest affinity for wheat chromosomes might be expected to resemble wheat most closely in staining reaction.

Sax (7) presents a reproduction of a photomicrograph of meiotic chromosomes of *Secale cereale* (his fig. 1), which shows the transversely barred or "banded" appearance seen by the writer in what he believed to be the rye chromosomes of the Arlington wheat-rye amphidiploid. The stage at which this condition may be seen presumably is the late first prophase of the heterotypic division.

Previous experiments with interspecific crosses in plants have shown that somatic gametes may be produced, as was first demonstrated by Rosenberg (6) in *Hieracium* sp. The occurrence of somatic gametes in  $F_1$  wheat  $\times$  rye hybrids was shown by the writer (1) in the production of 49-chromosome plants upon backcrossing to the wheat parent. This means that some of the gametes (ovules) of the  $F_1$  hybrid had 28 chromosomes. The probable cytogenetic origin of the Arlington Farm amphidiploid is suggested by its history. It was produced by crossing a wheat  $\times$  rye  $\times$  wheat plant with rye. It is reasonable to suppose that somatic gametes (ovules) also may be formed in a 49-chromosome plant, which when crossed with rye pollen will complete the diploid chromosome complement of the rye and thus produce an amphidiploid (zygote).

Judging by the proportion of somatic gametes (3 out of 10) found in  $F_1$  wheat  $\times$  rye hybrids which the writer (1) determined in the  $F_1$  backcross to wheat, it is possible that wheat  $\times$  rye  $\times$  wheat somatic gametes also may occur not too infrequently, so that the production of amphidiploids of the wheat-rye type would be greatly facilitated by crossing the backcross to one parent with the other parent.

### SUMMARY

A constant wheat  $\times$  rye hybrid, bred and selected by J. W. Taylor at the Arlington Experiment Farm, near Washington, D. C., in 1925, was found by the writer to be an amphidiploid. Fifty-six chromo-

somes were found in the root tips and 28 pairs in the pollen mother cells.

Wheat and rye chromosomes could not be distinguished in the somatic cells. In the pollen mother cells at first metaphase the rye bivalents were found to be larger than those of the wheat. In most cases they were less deeply stained, some were banded transversely, and all had a strong tendency to arrange themselves at the border or edge of the chromosome group.

This amphidiploid resulted from a cross of wheat  $\times$  rye  $\times$  wheat  $\times$  rye, which makes it probable that somatic gametes were involved in its origin both in the  $F_1$  wheat  $\times$  rye hybrid and in the  $F_1$  wheat  $\times$  rye  $\times$  wheat backcross.

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## PHYTOPHTHORA ROT OF SUGAR BEET<sup>1</sup>

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### INTRODUCTION

Phytophthora root rot of sugar beet (fig. 1), caused by *Phytophthora drechsleri* Tucker, was first noted in 1927 in the Cache Valley of northern Utah, causing most severe damage in an area about 18 miles in length between Smithfield and Wellsville. In 1928 and 1929 the disease was also found in the northern part of the Cache Valley in southern Idaho and near Ogden and Provo, Utah, and Rifle and Carbondale, Colo. During 1932 to 1934 the disease was found in the Sacramento Valley and delta section in California.<sup>2</sup> Tests made in 1927 and 1928 showed that the root size and sugar content of the sugar beet are reduced as a result of the disease. The present paper presents the results of a study of the disease, commenced in 1927.

### DISEASE FAVORED BY WET SOIL

In Utah the disease was confined mainly to the heavier, poorly drained Trenton clay soils in Cache Valley, although small areas of infection were found in low places in sandy loam soil. In the first field in which the disease was found, plants growing on a ridge elevated 1½ to 2 feet were free from the disease, while a high percentage of the plants in the rest of the field were diseased (fig. 2, B). The same condition was found in this field during the two following seasons (1928 and 1929).

In California the disease has been found only where irrigation water has accumulated and where the soil has been excessively wet at



FIGURE 1—Phytophthora rot of sugar beet, showing typical infection of basal part of the taproot, collected in the Sacramento River delta section Oct. 10, 1933

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<sup>2</sup> TUCKER, C. M. THE DISTRIBUTION OF THE GENUS PHYTOPHTHORA. Mo. Agr. Expt. Sta. Research Bull. 184, 80 pp. 1933. (See p. 9.)



FIGURE 2.—A, Longitudinal section of an advanced case of *Phytophthora* rot, showing tassellike mass of vascular strands at basal end of the root; B, portion of field near Logan, Utah, October 1929, showing the severity of the disease, the leaves having wilted and most of the plants having been killed; C, longitudinal section of diseased root from field in Utah showing blackish-brown band of tissue at the sharply defined advancing edge of the citrine-drab, water-soaked infected tissue; D, cross sections of field-infected roots showing the black line at the irregular margin of the infected tissue; E, cross section showing cavities formed within the infected tissue and sunken nature of the external surface of the lesion.

some time during the season. It is generally recognized by growers as a trouble associated with excess soil moisture and is sometimes called wet rot or water rot.

In an irrigation experiment carried out in 1929 in the field in the Cache Valley where the disease was first found, three well-separated plots of 25 rows each were given different amounts of irrigation water. All plots received an irrigation 1 week after thinning, after which plot A received a light irrigation in furrows once every 2 weeks, plot B, every 3 weeks, and plot C, every 4 weeks. In plot A, the most heavily irrigated, 67 percent of the plants were infected, in plot B, 42, and in plot C, 15 percent. This indicates clearly that the more frequent irrigation favored infection, and that proper irrigation practice should control the disease.

#### EFFECT OF PHYTOPHTHORA ROT ON YIELD AND SUGAR CONTENT

Approximately 500 acres of beets were infected in varying degree in the southern half of Cache Valley in September 1927. In many fields, the number of healthy plants was so reduced that the yield did not defray the cost of harvesting. Losses ranged from a trace to 90 percent in individual fields. By count, 60 percent of the plants in one field presented unmistakable symptoms. Diseased plants occurred singly or in groups interspersed among healthy plants. Various stages of rotting of the root, accompanied by different degrees of wilting of the foliage, were exhibited, but for the most part advanced symptoms predominated.

More detailed surveys in 1928 and 1929 showed that in excess of 1,000 acres of land were more or less infested. Heavy losses were frequently met with on new land, plowed and planted for the first time. Many diseased roots, from which the fungus was isolated, were found in factory yards throughout the Cache Valley, 1 to 2 months after digging.

Preliminary tests in 1927 and 1928 showed that the disease reduced root size and sugar content. In August 1929 a representative area was selected for sampling in each of four fields in the Cache Valley. Healthy and diseased beets were dug, in as close proximity as possible. Diseased beets having more than half of the root rotted were discarded. The samples were weighed and analyzed in the laboratory of the sugar factory. The results are presented in table 1.

TABLE 1.—*Effect of Phytophthora rot on weight and sugar content of sugar beets in fields in Utah, Aug. 31, 1929*<sup>1</sup>

Location of field	Condition of beets	Beets	Average weight per beet	Sugar	Purity <sup>2</sup>
		Number	Grams	Percent	Percent
Logan	Healthy	12	551.5	13.5	82.4
	Diseased	25	189.4	7.5	59.8
Field 1	do.	25	146.5	5.3	47.8
	Healthy	12	432.9	15.8	84.8
Field 2	Diseased	25	132.4	9.9	61.2
	do.	25	159.1	10.4	63.4
Field 3	Healthy	20	419.6	15.8	85.3
	Diseased	20	201.3	11.2	72.4
Smithfield	Healthy	20	544.3	17.0	84.1
	Diseased	30	266.5	11.6	72.1

<sup>1</sup> Analyses were made in the chemical laboratory of the Amalgamated Sugar Co. at Logan, Utah.

<sup>2</sup> Coefficient of purity is the ratio between the percentage of total solids and the percentage of sugar.

The average weight of the healthy beets was more than twice that of the diseased beets. The sugar in the diseased beets ranged from 5.3



to 11.6 percent, as compared with 13.5 to 17.0 percent in the healthy beets. The purity of the juice of the diseased beets ranged from 47.8 to 72.4 percent, as compared with 82.4 to 85.3 percent for the healthy beets.

Subsequent tests of the juice of samples of diseased beets collected during the remainder of the season from these and other fields gave even a greater reduction in sugar content and in purity so that at harvest the differences between healthy and diseased roots were even more striking.

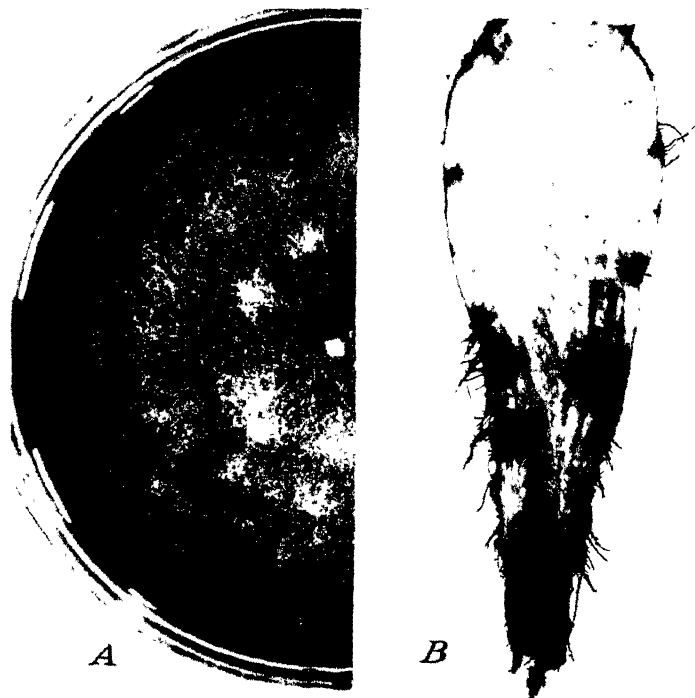


FIGURE 3 —A, Five-day-old culture of *Phytophthora drechsleri* on prune agar, pH 4.7, at 28° C., B, longitudinal section of field-infected root showing lateral lesions, some at the bases of the rootlets

#### SYMPTOMS OF THE DISEASE

In the early stages of the disease the leaves may wilt during the day and recover at night. Later, permanent wilting occurs and the plant is killed (fig. 2, B). Generally, infection occurs near the basal part of the root and the invasion progresses gradually upward involving all tissues of the root (fig. 1 and fig. 2, C, E). In roots inoculated in the laboratory, the affected tissues remain fairly firm and turgid (fig. 2, C), but under field conditions the affected parenchyma at the base of the root may soften and disintegrate, leaving only the vascular strands. This gives a frayed, tassellike, skeletonized appearance to the basal part of the fleshy taproot (fig. 2, A). Secondary or adventitious lateral roots often develop above the invaded region of the taproot and an ill-shaped, pronglike root is the result.

Often lesions first arise on the sides and sometimes near the upper part of the taproot (figs. 2, D and 3, B). Infrequently, infection occurs

at the tips or along the sides of the lateral roots or rootlets, with subsequent invasion of the taproot around the base of the infected lateral root or rootlet (fig. 3, *B*).

The lesions vary in size and shape and sometimes the entire root is involved. The predominant external color of the lesion ranges from the mummy brown to light seal brown of Ridgway,<sup>3</sup> tending toward blackish brown at the center.

When the root is cut, the infected tissues are found to be sharply delimited from the healthy tissues (fig. 2, *A, C, D*). Generally, color zones are visible in the lesion. The tissues first infected are deep brownish drab;<sup>3</sup> the central parts of the infected region are water-soaked and citrine drab to smoke gray, or buckthorn brown, or light buff to mummy brown; and the recently infected tissue at the advancing edge of the lesion as viewed on the cut surface is composed of a narrow band of blackish brown, sometimes separated from the healthy tissue by a narrow band of light buff (fig. 2, *C, D*). In the early stages the vascular bundles are conspicuous and light seal brown in color, but later this distinction may be lost. In the final stages the tissues are blackish brown (fig. 2, *A*). Small pockets or cavities are often formed (fig. 2, *E*) but are not filled with mycelium as in dry-rot canker caused by *Rhizoctonia solani* Kühn.

Microscopic examinations of stained sections of tissue from field-infected roots as well as roots inoculated in the laboratory show that the mycelium is, in general, intercellular, although certain cells are filled with mycelium. There was no evidence of any dissolution of the middle lamella.

#### ISOLATION OF THE CAUSAL FUNGUS

The causal fungus was readily isolated on prune agar, and usually pure cultures were obtained from tissue plantings from the advancing edge of the lesions. Isolations were made from more than 300 roots collected in 20 fields in Utah in 1927 and 1928. In not a single instance were other fungi or bacteria obtained (table 2). Good growth developed at 28° C. within 36 hours. The fungus was also isolated from infected lateral roots and lateral rootlets. Attempts to isolate the organism from healthy tissues of a diseased root, as well as from wilted and turgid petioles and leaves of diseased plants, gave negative results. The same fungus was isolated from diseased roots collected in subsequent years in Utah, and in 1932 in the Sutter basin and delta section in California.

TABLE 2.—Isolations of *Phytophthora drechsleri* from diseased sugar-beet roots in Utah

Location of field	Date of isolations	Roots	Roots yielding <i>P. drechsleri</i>	No growth
Logan		Number	Number	Number
Field 1.....	Aug. 3, 1927.....	50	43	7
Field 2.....	Sept. 5, 1927.....	30	26	4
Field 3.....	Aug. 22, 1928.....	132	94	38
Field 4.....	Aug. 8, 1928.....	100	87	13
Smithfield.....	Aug. 23, 1928.....	50	40	10
Total.....	.....	362	290	72

<sup>3</sup> RIDGWAY, R. COLOR STANDARDS AND COLOR NOMENCLATURE. 43 pp., illus. Washington, D. C. 1912  
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## THE CAUSAL FUNGUS

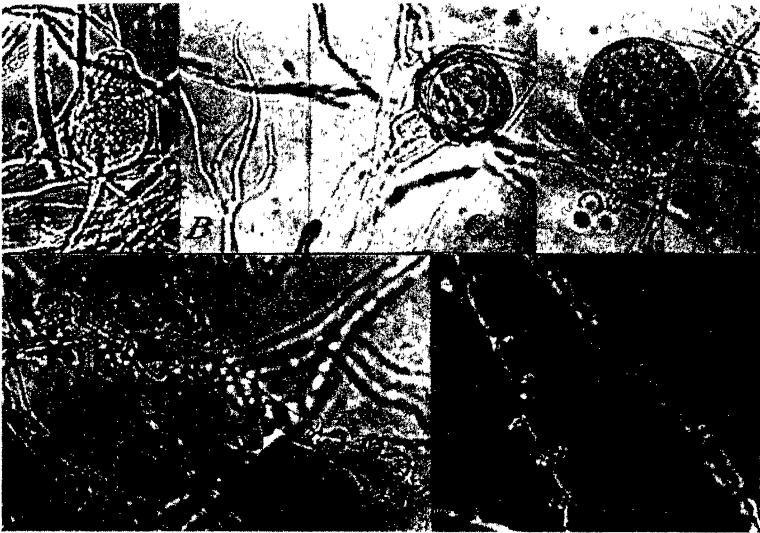
The causal organism was identified as *Phytophthora drechsleri* Tucker.<sup>4</sup> In young cultures on potato-dextrose agar the fungus developed a moderate amount of aerial mycelium with no reproductive organs, and on moist corn meal a white cottony mass of mycelium was present after 2 weeks. On prune agar the fungus produced a delicate, moderately dense mycelium with scant aerial growth (fig. 3, A). The fungus grew luxuriantly on oatmeal agar slopes, filling the space between the slope and the wall of the tube with a densely matted, tough, white mass of hyphae, but failed to develop reproductive bodies after 25 days at room temperature and produced only occasional oogonia after 2 months at 15° to 20° C. The hyphae were usually smooth and uniform in diameter, but occasionally they were gnarled and swollen, sometimes with tuberlike or vesicular growths, especially in old potato-dextrose agar cultures (pl. 1, D, E).

Oogonia with amphigynous antheridia developed sparingly in potato-dextrose agar cultures incubated 2 months at 15° to 20° C. They were thin-walled, hyaline to yellow or light brown, spheroidal with a narrow stalk completely enclosed by the antheridium (pl. 1, C). The oogonia varied in diameter from 27.2 $\mu$  to 44 $\mu$ , averaging 35.8 $\mu$ . The antheridia were borne on slender hyphae smaller than the usual vegetative strands. They were generally 10 $\mu$  to 14 $\mu$  in diameter and often distorted by pressure on the oogonium. The antheridium formed a collar around the oogonial stalk and was closely oppressed to the inflated portion of the oogonium where it remained until the oogonium disintegrated (pl. 1, C).

The oospores developed singly in the oogonia. They were smooth, thick-walled, yellow to light brown, spherical, only partially filling the oogonial cavity (pl. 1, C). In old cultures the oogonial wall became crumpled and finally disintegrated, liberating the oospore. The oospores were 23.6 $\mu$  to 35.2 $\mu$  in diameter, averaging 28.6 $\mu$ . Germination was not observed.

When tufts of mycelium from oatmeal-agar cultures 3 weeks old were transferred to Petri's solution sporangia developed after 2 days. The sporangia were hyaline to faintly yellow, thin-walled, obpyriform, acrogenous on slender sporangiophores, nonpapillate but provided with a hyaline lens-shaped thickening at the apical end, 24 $\mu$  to 56 $\mu$  by 22.8 $\mu$  to 40 $\mu$ , averaging 38.6 $\mu$  by 26.7 $\mu$  (pl. 1, A). Germination occurred by germ tubes or by the development of a variable number of motile zoospores, 10 $\mu$  to 12 $\mu$  in diameter, which were fully differentiated within the sporangium. The lenticular thickening in the apex of the sporangium was pushed out or dissolved and the zoospores escaped, soon became quiescent, rounded up, underwent thickening of the wall, and finally germinated by a slender germ tube, the usual type of development among species of *Phytophthora*. Following zoospore discharge, the sporangiophore often resumed growth, pushing through the base of the evacuated sporangium and growing out through the apical aperture (pl. 1, B). A new sporangium was often formed just beyond or occasionally within the old wall. Sometimes the process was repeated, leaving the walls of two evacuated sporangia enclosing the sporangiophore bearing a sporangium at the tip.

<sup>4</sup> TUCKER, C. M. TAXONOMY OF THE GENUS *PHYTOPHTHORA* DE BARY. Mo. Agr. Expt. Sta. Research Bull. 153, 203 pp., illus. 1931. (See p. 188.)



*Phytophthora drechsleri*, Tucker: .1, Nonpapillate sporangium, with a refringent lens-shaped thickening at the apex, developed on hyphae from an oatmeal agar culture after 2 days in Petri's solution,  $\times 430$ ; B, an empty sporangium with the sporangiophore emerging from the orifice following resumed proliferation subsequent to discharge of the contents of the sporangium,  $\times 430$ ; C, at the left, oogonium, oospore, and antheridium and, at right, oogonium and antheridium, prior to the development of the oospore, the antheridium forming a collar around the narrow oogonial stalk,  $\times 430$ ; D and E, gnarled and tuberculate hyphae from a potato-dextrose agar culture 2 months old.

## TEMPERATURE RELATIONS OF THE FUNGUS IN CULTURE

The relation of temperature to growth of the mycelium was determined for two cultures, one from Utah and one from California. For this purpose, large test tubes 20 cm in length, provided with a dam at the open end, made by heating the glass and indenting one side, were used. Prune agar (15 cc) of pH 4.7 was placed in each tube and allowed to solidify with the tube in a horizontal position. The dam prevented the escape of the melted agar.

These tubes were inoculated near the mouth with small squares of prune agar with mycelium from Petri-dish cultures 5 days old. After an initial exposure at room temperature (20° to 23° C.) for 24 hours, the position of the advancing edge of the colony was marked with a wax pencil on each tube, and three tubes of each culture were placed in a horizontal position in thermostatically controlled, electric incubators at 3°, 8°, 12°, 16°, 19°, 22°, 25°, 28°, 31°, 34°, 37°, and 40°. The daily growth of the mycelium beyond the wax-pencil mark was measured.

There was no marked difference between the Utah and California cultures. No growth occurred at 3°, or at 37° or 40° C. The average daily growth at 8° was 2.3 mm; at 12°, 5 mm; at 16°, 6.6 mm; at 19°, 7.3 mm; at 22°, 7.7 mm; at 25°, 9.6 mm; at 28°, 10.0 mm; at 31°, 10.0 mm; and at 34°, 8.7 mm. In another test on corn-meal agar in Petri dishes, the average diameter of the colony after 96 hours was 44 mm at 20°, 48 mm at 25°, 54 mm at 30°, and 49 mm at 35°. The lowest temperature for growth, therefore, is between 3° and 8°, the optimum, 28° to 31°, and the maximum between 35° and 37°. These characteristics, particularly the tolerance of high temperatures, are in agreement with those reported by Tucker<sup>11</sup> for the type culture.

## RELATION OF HYDROGEN-ION CONCENTRATION TO GROWTH OF THE FUNGUS

Prune agar, adjusted to different pH values between pH 3.6 and pH 8.4 and placed in the large test tubes as previously described, was used to determine the relation of hydrogen-ion concentration to the growth of the fungus. The cultures were incubated at 28° C., and the daily growth increments over a period of 12 days were measured.

Good growth occurred at all the pH values tested, varying from 7.6 mm per day to 12.7 mm per day, with no indication that the limits of hydrogen-ion or hydroxyl-ion tolerance were being approached. The daily rate of growth at pH 3.6 was 8.3 mm; at 3.9, 9.6 mm; at 4.2, 12.7 mm; at 4.5, 9.9 mm; at 4.8, 10.0 mm; at 5.1, 10.0 mm; at 5.4, 9.7 mm; at 5.7, 10.2 mm; at 6.0, 9.9 mm; at 6.3, 10.2 mm; at 6.6, 10.5 mm; at 6.9, 9.8 mm; at 7.2, 10.0 mm; at 7.5, 8.3 mm; at 7.8, 7.6 mm; at 8.1, 10.6 mm; and at 8.4, 10.7 mm.

## INFECTION OBTAINED ON WOUNDED AND UNWOUNDED PLANTS IN THE FIELD

For preliminary field tests in Utah in 1927 healthy vigorous plants were selected, and the soil was carefully excavated from one side of each root on August 15. The exposed side of the root was wiped with wet cotton and then with alcohol. In one series of inoculations, punc-

<sup>11</sup> TUCKER, C. M. See p. 189 of citation mentioned in footnote 4.

tures were made with a flamed needle well below the middle of the fleshy taproot, and mycelium from cultures on prune agar or sterilized oats was placed in contact with the injured periderm and the soil replaced. In a second series the inoculum was applied to the unwounded surface of the root. Control plants were treated similarly in each series except that sterile culture medium was substituted for the inoculum.

All plots were given two light irrigations between the time of inoculating and harvesting. Marked wilting developed on most of the inoculated plants within 30 days, and when the beets were dug on September 20, 82 percent of the 160 roots inoculated with wounds were infected and 41 percent of the roots inoculated without wounding were infected. The control plants remained healthy. The infected roots bore typical lesions, those on the wound-inoculated roots being the larger. The fungus was reisolated from a large number of the infected roots and in inoculations made on 10 plants on September 28 again proved pathogenic. Additional inoculation tests were made in Utah in the summers of 1928 and 1929. The results together with those of 1927, are summarized in table 3.

On August 8, 1929, healthy beets in the field were inoculated with small blocks of naturally infected beet tissue. Of 20 beets wounded by needle punctures, 17 were infected when examined on September 22, and of 20 beets inoculated without wounding, 13 were infected. The 10 control plants for each method remained healthy. The fungus was reisolated from the infected beets (table 3).

On August 8, 1934, plants were inoculated in the field at Clarksburg, Calif., by placing mycelium grown on cracked wheat in the soil alongside the unwounded roots. The beets were irrigated several times and were dug on September 20. Infection had occurred, and the fungus was reisolated.

#### INFECTION OBTAINED ON WOUNDED AND UNWOUNDED ROOTS IN THE LABORATORY

In Utah, in 1927, cultures of the fungus were used in crown inoculations of healthy topped beets. All beets were washed in tap water, treated for 10 minutes in a 1-to-1,000 solution of mercuric chloride, and rinsed in three changes of sterile distilled water. A thin slice of crown tissue was removed with a flamed knife to expose fresh tissues free from cork cells. The inoculum, mycelium from 8-day cultures on prune agar, was placed on the cut surface and covered with moist absorbent cotton held in position by means of adhesive tape. All beets were placed in individual glassine bags and stored in a root cellar at a temperature ranging from 5° to 10° C. After 35 days, 24 of the 28 inoculated beets were found to be infected, as proved by reisolations, and the amount of decay in individual beets ranged from 15 to 70 percent by volume. The 10 controls treated with sterile prune agar were not infected.

Inoculation tests were made on the unwounded surface of sugar beets in the laboratory at Berkeley in January 1933, with one Utah and three California cultures of the fungus. Beets were thoroughly washed in tap water, and 95-percent alcohol was applied to the periderm where the inoculations were to be made. Mycelium grown on prune agar was inserted in two wells in each root made by removing

small cubes of tissue with a flamed scalpel, and covering with adhesive tape. Controls were treated with sterile prune agar. All were incubated in moist chambers at laboratory temperatures (20° to 24° C.) for 20 to 45 days. For moist chambers, use was made of 5-gallon tin cans with pie tins for covers; each can was provided with a wire-mesh platform to prevent contact of the roots with the water in the bottom of the can. Of the 44 roots inoculated with the California cultures, 39 were infected, and of the 39 inoculated with the Utah culture 30 were infected. The fungus was reisolated from all of the infected roots. None of the 21 controls became infected. The cultures showed no difference in virulence.

TABLE 3.—Results of inoculations on sugar beets with *Phytophthora drechsleri* in Utah, 1927-29

Type of inoculum	Method of inoculation	Beets inoculated	Beets infected	Beets from which <i>P. drechsleri</i> was reisolated
		Number	Number	Number
Mycelium on agar.....	{Needle.....	170	144	69
	{No injury.....	130	62	73
Mycelium on oats.....	{Needle.....	80	60	40
	{No injury.....	40	16	34
Infected beet tissues.....	{Needle.....	20	17	9
	{No injury.....	20	13	10
Control, sterile agar.....	{Needle.....	105	0	-----
	{No injury.....	105	0	-----
Control; sterile oats.....	{Needle.....	60	0	-----
	{No injury.....	60	0	-----
Control, healthy beet tissues.....	{Needle.....	10	0	-----
	{No injury.....	10	0	-----

Unwounded roots were also successfully inoculated. On March 14, 1933, eight roots harvested in November and kept in cold storage were inoculated by placing a half-inch square from a prune-agar culture on a clean, sterilized but unwounded portion of the root surface. The roots were incubated in moist chambers at room temperature and within 2 weeks 5 of the 8 inoculated beets were typically diseased and the fungus was reisolated. The controls, on which sterile agar had been placed, were not infected.

#### BEEET SEEDLINGS SUSCEPTIBLE TO INFECTION

No infection of sugar-beet seedlings by *Phytophthora drechsleri* has been observed under field conditions. Many diseased seedlings have been cultured, but this fungus has never been obtained. Presumably soil temperatures during the seedling stage of this crop are below the optimum for this fungus.

Laboratory tests showed, however, that seedlings were readily infected at higher temperatures. For these tests, small paraffined paper cups, 3 inches deep, were filled with sterilized river sand. Prune-agar cultures of the fungus were incorporated with the surface soil in the cups and 10 surface-sterilized, sugar-beet seed balls were planted in each cup. All cups were irrigated uniformly with sterile, distilled water and placed in incubators at constant temperatures of 25°, 28°, 31°, and 34° C. The seed balls failed to germinate at 34°.

Abundant infection of the seedlings occurred in 9 days at the other temperatures, as is shown in table 4. Infected seedlings exhibited damping-off symptoms, and the cortical tissues were water-soaked in appearance. Some seedlings attained a height of 1½ inches before being killed, but most of them were attacked in earlier stages of growth. Some infected seedlings barely emerged, and it was apparent that many died before emergence. The fungus was reisolated from practically all of the infected seedlings. Normal seedlings developed in the controls.

TABLE 4—Infection of sugar-beet seedlings grown in sterilized inoculated sand at constant temperatures

Temperature (°C)	Source of culture	Seedlings	Seedlings diseased	Temperature (°C)	Source of culture	Seedlings	Seedlings diseased
		Number	Number			Number	Number
25-----	California.....	39	32	31-----	California.....	21	12
	Utah.....	27	25		Utah.....	11	6
	Controls.....	58	0		Controls.....	28	0
	California.....	19	18				
28-----	Utah.....	6	6				
	Controls.....	51	0				

#### PATHOGENICITY OF FUNGUS TO CERTAIN OTHER HOSTS

Tucker<sup>12</sup> found *Phytophthora drechsleri* pathogenic to wounded apple, eggplant fruits and tomato fruits, wounded eggplant, papaw, and tomato seedlings, and potato tubers. Wound inoculations have been successful on the roots of garden beets, carrots, turnips, parsnips, and potatoes. The identity of the fungus was confirmed by reisolations. In garden beets the diseased tissues were black and firm, in carrots water-soaked and moderately firm except in advanced stages, and in parsnips and turnips gray, water-soaked, and soft and mushy after 7 days. In potato tubers the infected tissues were softened and rubbery after 5 days, and cut surfaces when exposed to the air developed a light pink discoloration within 15 minutes which darkened within an hour and changed to black. A definite line separated diseased from healthy tissues.

Wound inoculations with the fungus were successful in watermelons and citrons. Large brown lesions developed on the surface within 48 hours at room temperature, and within 10 days the fruits had softened and were almost completely rotted.

Sixteen unwounded ripe tomato fruits were inoculated by placing small squares of an agar culture on the skin at the blossom end and holding them in glass, moist chambers at room temperature. All became infected, some in 6 days, and showed softened, water-soaked lesions, with irregular margins.

The pathogenicity of the fungus was also tested by inoculations on unwounded apple, eggplant, summer squash, green tomato, and green pepper fruits. The apples and green tomatoes were not infected. Eggplant fruits were readily invaded, and after 5 days chestnut-brown lesions 5 cm in diameter developed. The tissues were softened and discolored to a depth of 1 to 1½ inches. Two of the twelve green peppers inoculated and 7 of the 16 summer squashes were infected.

<sup>12</sup> TUCKER, C. M. See pp 69 and 189 of citation mentioned in footnote 4.



Wound inoculations were made on apples, summer squashes, green tomatoes, and green peppers. After surface sterilization, each fruit was punctured 10 times with a flamed needle in an area one-half inch in diameter. A small square of agar from a culture was then placed on the punctured area. Three days later all the apple fruits were slightly invaded, the summer squashes were half decayed, the peppers were all infected, and the green tomatoes were almost completely softened. All controls were healthy at the end of the test period.

#### SUMMARY

Phytophthora rot of the sugar beet has been found in Utah, Idaho, Colorado, and California in poorly drained soils or areas where irrigation water has accumulated. Infection is favored by excessively wet soil. Yield and sugar content are greatly reduced.

The basal end of the taproot is usually rotted, although infection may occur along the side of the taproot, especially where rootlets are attached. In the field the disease causes wilting of the leaves during the day and ultimate death of the plant. In the early stages the infected root tissues as seen on the cut surface are water-soaked and light brown, with a thin blackish-brown zone at the advancing face of the lesion. Later the parenchyma tissues may disintegrate and leave a tuft of fibers at the tip of the root.

The disease is caused by *Phytophthora drechsleri* Tucker, of which a description is given. The fungus grows at temperatures ranging from 8° to 35° C., with its optimum at 28° to 31°. It grows well throughout a wide pH range.

With mycelium as inoculum, infection was readily obtained on both wounded and unwounded roots of plants in the field and also on harvested roots in the laboratory.

The fungus caused damping-off of sugar-beet seedlings grown in inoculated soil at laboratory temperatures.

In the laboratory wound inoculations with mycelium were successful on the roots of garden beet, carrot, turnip, and parsnip, on potato tubers, and on apples, summer squash fruits, green peppers, and green tomatoes. Inoculations without wounds were successful on eggplant fruits, green peppers, and summer squash fruits, but not on apples or green tomatoes.

# HISTOLOGICAL STUDY OF TISSUES FROM GREENHOUSE TOMATOES AFFECTED BY BLOTCHY RIPENING<sup>1</sup>

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## INTRODUCTION

The appearance and quality of greenhouse tomatoes (*Lycopersicon esculentum*) maturing in late spring and early summer are often impaired by red and colorless markings, commonly called blotchy ripening. In some cases the percentage of irregularly ripened fruits is less than 1 percent, while in others it may be as high as 50 percent. Investigations as to the nature of the disorder and methods for its control are being conducted at the Michigan Agricultural Experiment Station. This paper presents, as a part of these investigations, the results of a histological study of the tissues from blotchy-ripened fruits.

## EXTERNAL APPEARANCE OF BLOTCHY RIPENING

Blotchy ripening is primarily a disorder of the spring crop of greenhouse tomatoes maturing in May, June, and July and is of little consequence to the fall crop maturing from October to January. Throughout the North Central States it rarely occurs under field conditions, although a few fruits showing all the characteristics of the disorder were observed during the period of the severe drought of 1934.

In affected fruits areas of the outer wall<sup>3</sup> (pericarp) fail to develop and color normally. The individual blotch may be relatively large, involving from 25 to 50 percent of the entire surface of the fruit, or may be so small that it is not readily distinguishable. A fruit may show only one small blotch or several large blotches; large and small blotches may be formed on the same specimen. No sharp line of demarcation separates the green or white areas from the normal red portions; they merge gradually. Though these areas may appear on any portion of the fruit, they generally radiate from the pedicel attachment. Blotchy fruits in which all portions of the fruits are involved are shown in plate 1. As the fruits approach maturity these areas remain hard and green; and as ripening proceeds further, they assume a waxy or glassy appearance which may be accompanied by a sunken or pitted condition.

The vascular bundles lying beneath these clear, glassy blotches invariably appear to be brown or black and necrotic. This condition may be observed through the epidermis in many fruits. An affected area may be confined to one of the furrows formed above the inter-locular walls of the fruit; such areas have been frequently observed immediately above the main bundle of a carpel. Transverse cuts made through a blotchy fruit (pl. 2, A) show that only the outer wall is affected. Usually there are small discolored regions in the center

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<sup>2</sup> The writers are grateful to F. C. Bradford for helpful suggestions and assistance as to the methods employed and in the preparation of the manuscript.

<sup>3</sup> The term "outer wall" as used in this paper does not include the partitions between the locules.

of the blotch which have the appearance of necrotic vascular bundles. In severe cases cavities may be associated with the affected tissues. The discolored portions are found only in the distinct blotches and do not occur in the normally ripened portions. Longitudinal cuts (pl. 2, *B*) reveal the apparently necrotic bundles and associated tissues even more strikingly than do the transverse ones. As shown in the longitudinal cuts this appearance of broken-down tissues is confined entirely to the blotchy areas.

The disorder is apparently restricted to practically mature fruits. Fruits in all stages of development have been dissected and examined for evidences of blotchiness. No immature fruit has shown any detectable signs of developing into blotchy fruit. The first evidences of blotchiness have been observed only after the fruit begins to develop color, which is usually from 2 to 5 days before it is ready to harvest.

#### LITERATURE REVIEW

Bewley and White (2)<sup>4</sup> attempted to dissect tomato fruits so as to expose the vascular system, but "owing to the delicate nature of the individual strands", abandoned this method and adopted clearing methods. They carefully removed the skin of a half-ripe fruit, scraped away the superficial tissue, and exposed the external portion of the vascular system. They presented drawings showing that the veins radiate from the stem end, and that in the proximal half of the fruit there are few cross connections, while in the distal half the veins anastomose to form an intricate network. By clearing a fruit thus prepared in xylol following alcohol they obtained "a good idea of the vascular system of the entire fruit."

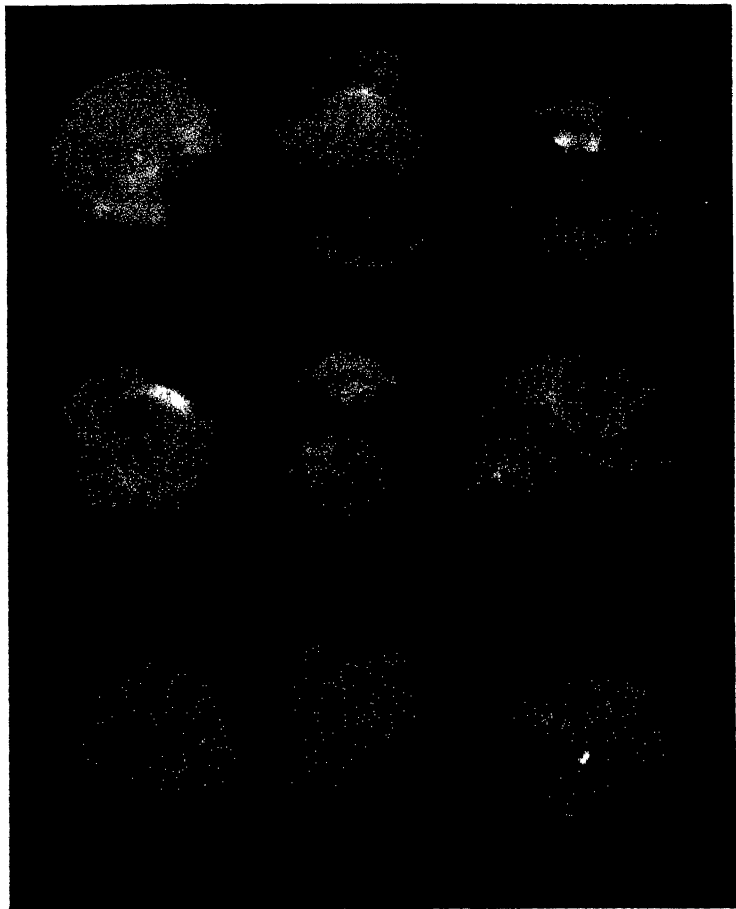
Their examinations revealed certain differences between the vascular bundles of blotchy fruits and those of healthy fruits. "The bundles of blotchy fruits seemed somewhat thicker than those of healthy fruits, owing to the spongy nature of the cells immediately surrounding them. Necrosis of the bundles frequently occurs" (2, p. 325). Drawings are shown of necrotic bundles appearing beneath distinct blotches. In another illustration they showed an affected bundle with an adjacent group of "corky cells" and stated that "such cells occur frequently in blotchy fruits." By means of a hand lens they observed "the presence of gaps or canals in the parenchyma bordering the necrotic bundles." The walls of these cells were brown and necrotic. Although no mention is made of the fact, their drawings indicate that plugging of the tracheae accompanies the necrosis.

Bewley and White (2, p. 337) from their results over a 5-year period (1921-25) concluded that "blotchy ripening of tomato fruits is the result of malnutrition in respect of potash and nitrogen", and "may be reduced to less than 1 percent. by suitable applications of sulphate of potash and sulphate of ammonia." However, they did not attempt to connect "the necrosis of the bundles" with these nutrient deficiencies.

Seaton's investigations of the disorder <sup>5</sup> (12) indicate that blotchy ripening is not a nutritional disorder arising from soil conditions. He has advanced the hypothesis that the withdrawal of water from the

<sup>4</sup> Reference is made by number (italic) to Literature Cited, p. 224.

<sup>5</sup> SEATON, H. L. INVESTIGATIONS ON BLOTCHY RIPENING OF GREENHOUSE TOMATOES. 1933. (Unpublished thesis, Mich. State Col.).

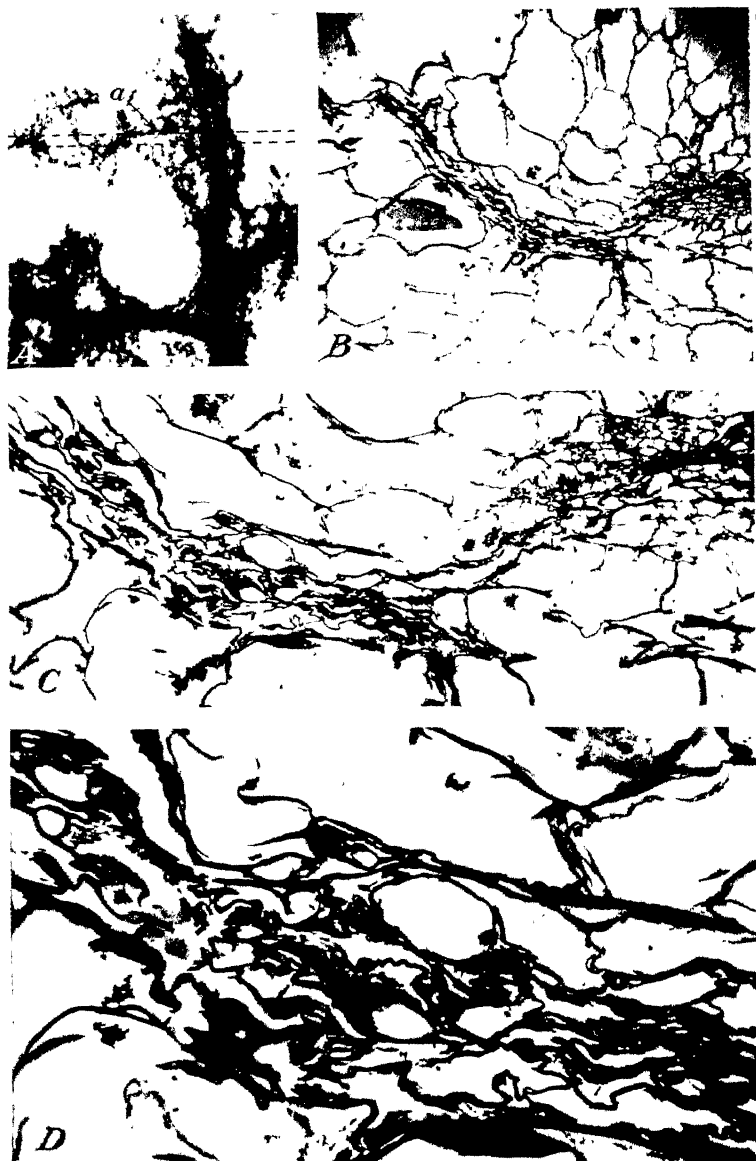


Tomatoes showing blotchy ripening. The glassy areas appear on all parts of the fruit but are less abundant in the styler region. Some of the blotchy areas show a sunken or pitted condition, whereas dark, discolored tissues adjacent to the bundles appear in others.

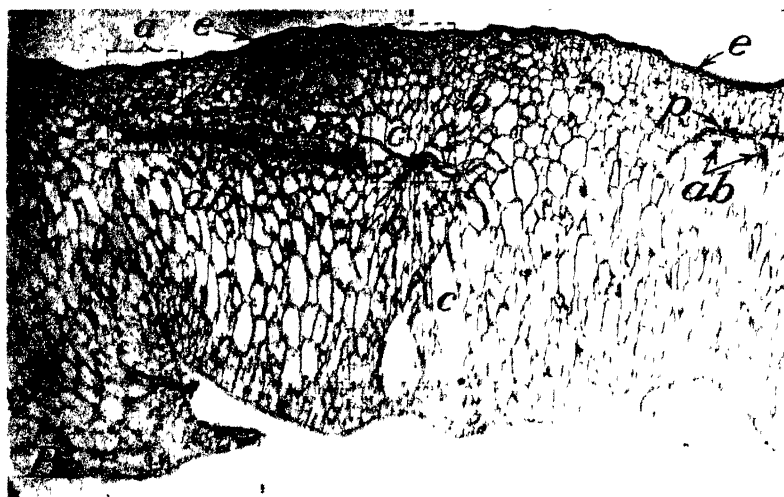
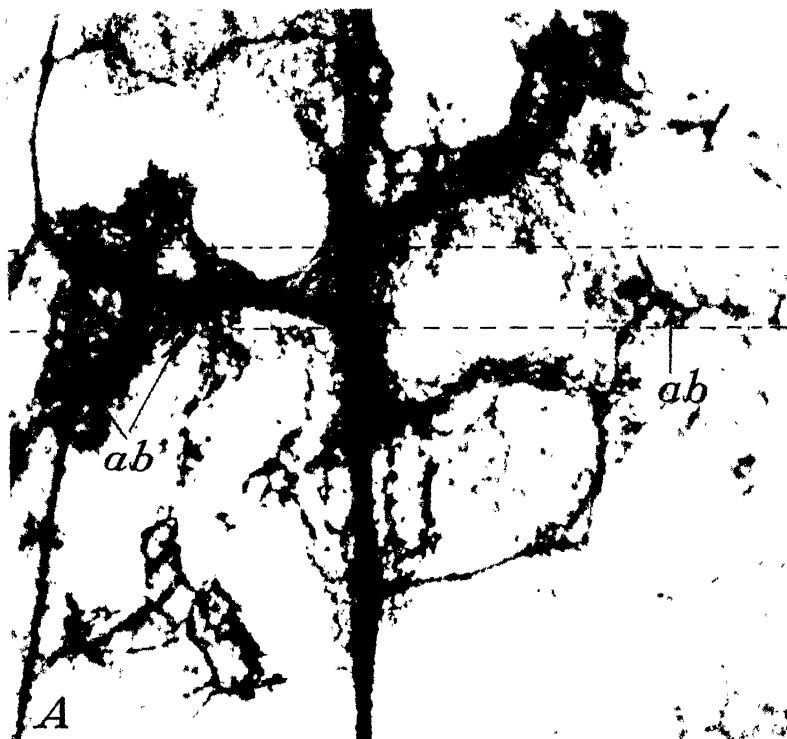




4, Blotchy ripened fruits cut transversely. Only the outer fruit wall is involved. The small dark areas in the blotches are affected tissues adjacent to the vessels.  $\times \frac{1}{2}$ . B, Fruits cut longitudinally to show the position of the affected tissues in the blotchy areas. No discolored tissues are apparent in the normal pericarp.  $\times \frac{1}{2}$ . C, A cleared section from a normal fruit wall near the styler region. One large carpellary bundle and numerous small anastomosing veins are shown.  $\times 5$ . D, Same as C, from an area near the stem end. The anastomosing veins are less numerous in this region.  $\times 5$ . E, Transverse section of a large carpellary bundle from a normal pericarp. This bicollateral bundle is typical. Note the large parenchyma cells (p) of the vascular ray.  $\times 125$ . F, Longitudinal section of a bundle from an unaffected fruit wall. The spiral thickened tracheae (tr), the adjacent phloem (ph), and the surrounding parenchyma (p) are shown.  $\times 125$

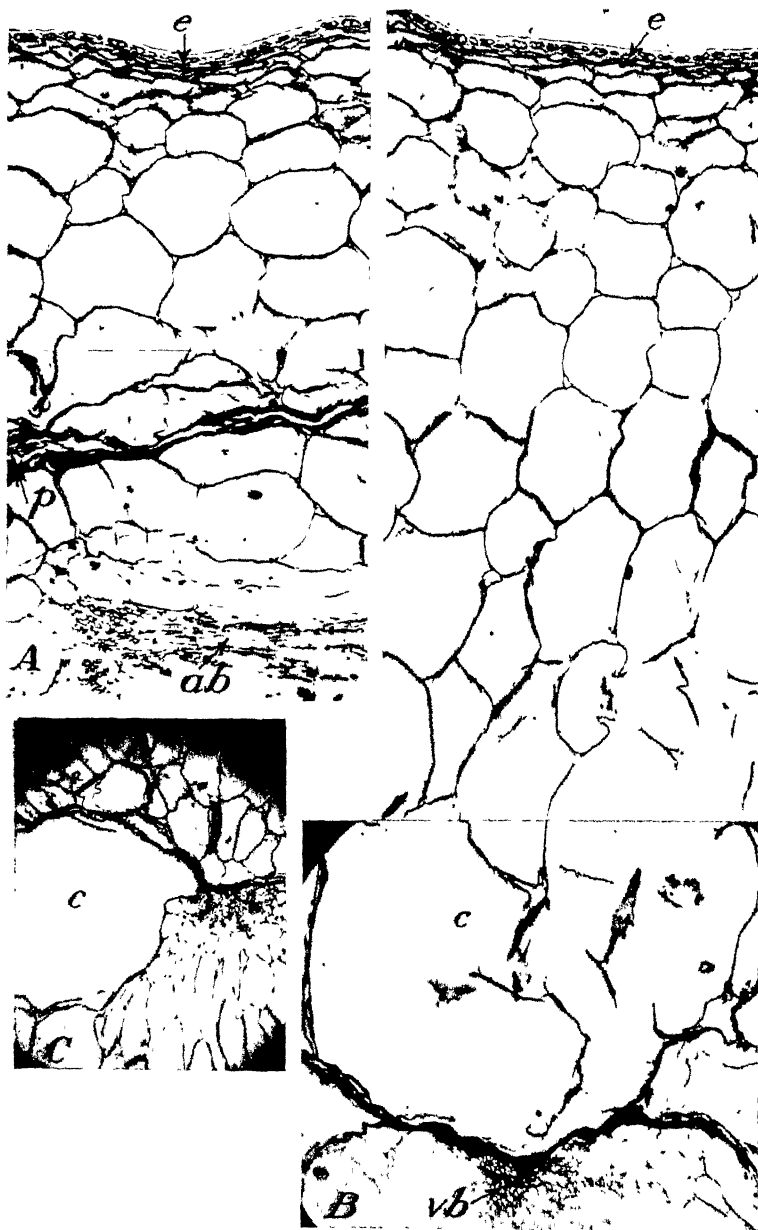


4. External view through the epidermis of a blotchy fruit. The discolored tissues appear to surround and involve the large bundle and the anastomosing veins. The broken lines (*a*) indicate the position of the section shown in *B*.  $\times 10$ . *B*, Transverse section from area indicated in *A*, *a*. Note the large vascular bundle (*vb*) and the broken down parenchyma tissue (*p*) which lies between the anastomosing bundle shown in *A* and the epidermis.  $\times 50$ . *C*, Identical with *B*. The vessels of the bundle appear unaffected and the discolored areas are collapsed parenchyma.  $\times 145$ . *D*, Identical with *C*. The distorted cell walls and cell contents which make up the dark areas are shown.  $\times 450$ .



*A*, View through epidermis of a blotchy fruit taken in the region of a carpellary bundle. Normal areas may be seen among the discolored portions which surround the anastomosing bundles (*ab*). The broken lines indicate the position of the cross section shown in *B*.  $\times 10$ . *B*, Transverse section of region indicated in *A*. Note the wavy appearance of the epidermis (*e*) with sunken portions above regions where the underlying parenchyma (*p*) has collapsed, while above the unaffected areas and where cavities (*c*) appear it is unaltered. The dark collapsed parenchyma tissues (*p*) are located between the epidermis (*e*) and the bundles (*ab* and *b*). See plate 5 for details of the marked portions *a* and *b*.  $\times 10$ .





*A*, Region indicated as *a* in plate 4. *B*, The epidermal cells (*e*) and the several underlying layers appear normal with the dark band resulting from the collapse of several layers of parenchyma (*p*) immediately above the anastomosing bundle (*ab*). The bundles show no evidences of necrosis or plugging, and apparently the cells subjected to break-down lie between the bundles and the epidermis.  $\times 75$ . *B*, Region indicated as *b* in plate 4. *B*, Compare the thickness of this area with that shown in *A*, where a collapse of cells is apparent. The large cavity (*c*) seems to be made up of dismembered cell walls, some of which have collected around the vessels of the bundle. As in *A* the broken-down tissues are located between the epidermis (*e*) and the bundle (*vb*).  $\times 75$ . *C*, Same bundle as shown in lower portion of *B*, but from a section taken above that shown in *B*. The vessels are unaffected and the dark areas are made up of distorted walls and cell contents of the parenchyma which once filled the cavity (*c*).  $\times 75$ .

fruits during periods of excessive transpiration, 3 to 5 days before the fruit ripens, results in the break-down of the tissues involved in the blotchy areas.

#### MATERIALS AND METHODS

The normal and blotchy-ripened fruits which furnished the material for this investigation were grown in the experiment station greenhouses at East Lansing in 1933 and 1934. Samples were collected each year in June and were about equally divided between fruits of the Grand Rapids variety and fourth- and fifth-generation hybrids between Ailsa Craig and Marglobe. In 1933 comparatively small portions of the outer fruit wall from distinct blotches were excised from freshly harvested material; but in 1934 much larger portions were taken, in some cases as much as one-fourth of the entire outer wall being used. The epidermis was removed from some samples and allowed to remain intact on others. In 1933 the material was killed and fixed in alcohol-formalin-acetic acid killing solution, embedded in paraffin, sectioned, and stained with Delafield's haematoxylin. In 1934 the samples were handled in the same manner, except that the large sections, after being cleared in xylol, were photographed with transmitted light in that liquid in an especially designed cell. After the gross photographs (pls. 2, *C* and *D*; 4, *A*; and 6, *A*), at a magnification of 5 and 10 diameters, were examined, the portions indicated in the plates were dissected from the cleared samples, embedded in paraffin, sectioned, and stained in the same manner as the 1933 material.

#### STRUCTURE AND APPEARANCE OF THE PERICARP

##### THE PERICARP FROM NORMAL FRUITS

When the outer fruit walls are cleared in xylol following alcohol, the vascular anatomy of the pericarp may be followed easily. Several large carpellary bundles radiate from the corky abscission layer, extend around the fruit, and converge again at the styler scar. Relatively large branches from the main carpellary bundle are found in the furrows outside the interlocular walls and smaller anastomosing veins from these extend into these walls (pl. 2, *B*). In the outer carpellary walls other branches of the main bundle appear and extend with it to the styler scar. In the pericarp near the pedicel attachment and extending to the region of the greatest diameter of the fruit few connecting veins are present between the main bundle and the large branches (pl. 2, *D*). However, from the region of the greatest diameter to the styler scar the bundles anastomose freely and form an intricate network of connecting veins (pl. 2, *C*). Thus the areas between the bundles and veins are larger, and more of their parenchymatous cells are further removed from bundles in the lower half of the ovary wall than in the upper half. These observations are, in general, in agreement with those of Bewley and White (2) and the observations by Cooper (4) on the anatomy and development of the tomato flower.

Histological examinations of the pericarp from mature fruits (pls. 4, *B*; and 5, *A* and *B*) show that the outer epidermis is made up of small, heavily cutinized polygonal cells. The inner epidermis is more delicate than the outer and shows little or no cutinization. Only the

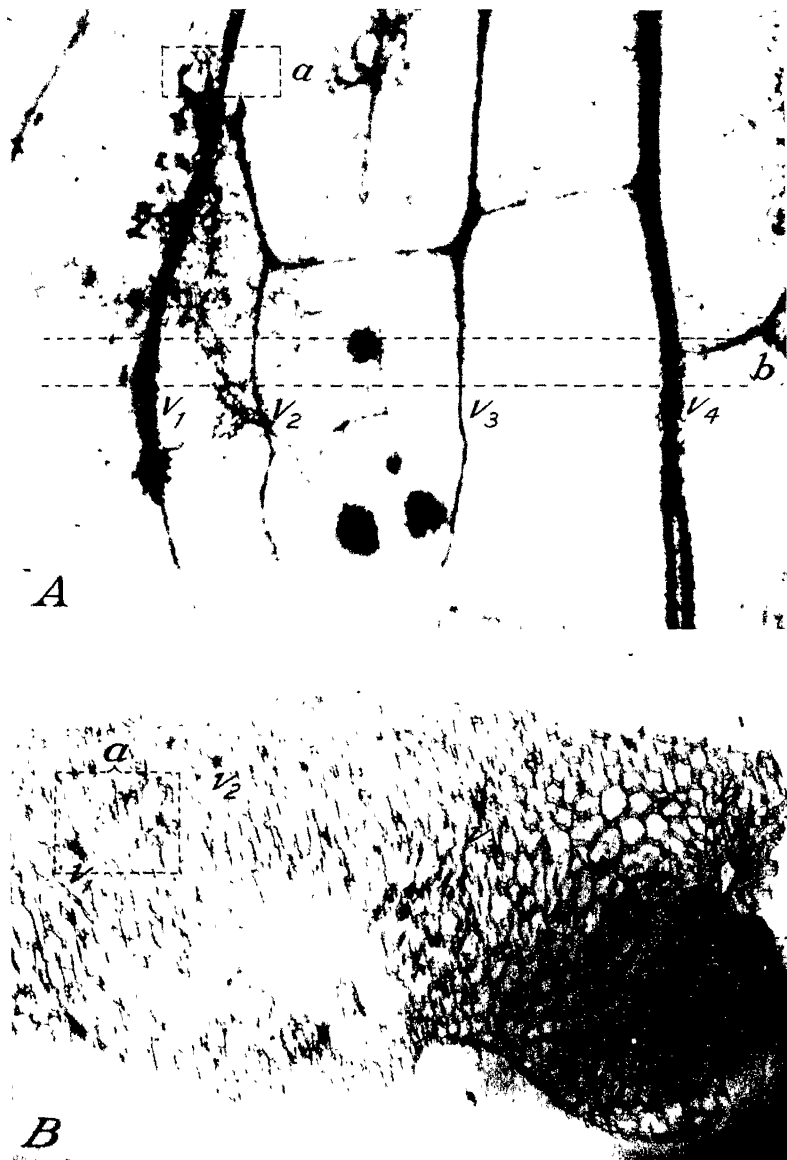
outer and the inner epidermis and the fleshy layer between the two are distinct. The several layers of parenchyma cells constituting the fleshy layer adjoining the epidermal layers are smaller and have slightly thicker walls than those of the center, where the cells are greatly extended and the walls are excessively thin and delicate at maturity. Inter-cellular spaces of any appreciable size are not present. Throughout the center of the fleshy layer, bicollateral vascular bundles, dissected with vascular rays of large, spongy parenchyma occur (pl. 2, *E*). In longitudinal section the tracheae of these bundles are seen to be the spirally thickened type (pl. 2, *F*).

#### THE PERICARP FROM BLOTCHY FRUITS

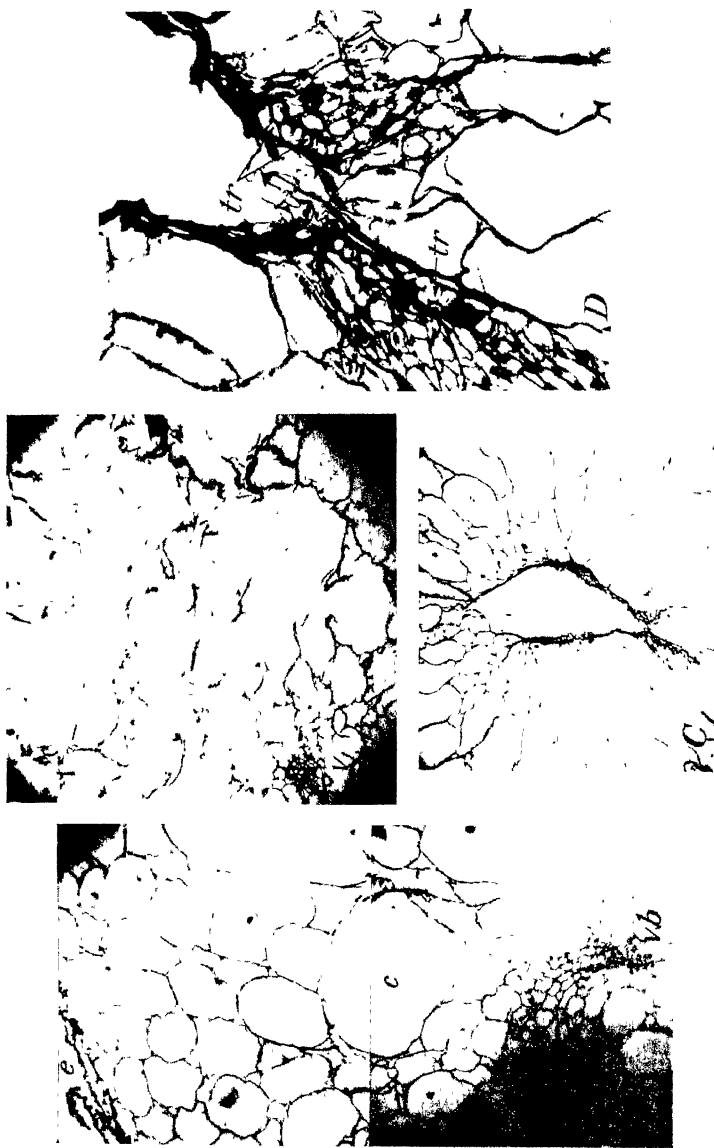
In cleared sections from blotchy fruits large dark areas attendant to the bundles and their branches are apparent, while less dense, slightly diffused areas of similarly affected tissues appear between the bundles (pls. 4, *A* and 6, *A*). Under low magnification the bundles in blotchy regions seem to be enlarged throughout the extent of the blotch and give the appearance of being necrotic and functionless, as Bewley and White (2) reported from their observations with a hand lens. However, when carefully prepared sections are examined under higher magnification, the actual conditions of the vascular elements and surrounding tissues appear quite different.

An external view through the epidermis of a blotchy fruit (pl. 3, *A*) illustrates the deceptive appearance of the necrotic and enlarged bundle and the anastomosing veins surrounded by affected tissue. In a transverse section of this same area (pl. 3, *B*) at higher magnification, the large bundle appears to the right of the field and the band of discolored tissue in the center is from immediately below the anastomosing bundle. The elements which appeared plugged at lower magnification are in reality open and unaffected, with some broken-down vascular ray parenchyma between them. The band of dense material, shown under high power (pl. 3, *C* and *D*), is clearly composed of collapsed parenchyma cell walls and the cell contents from the center of the fleshy layer of the ovary wall. The arrangement of the collapsed cells seems to indicate that they were subjected to mechanical stresses.

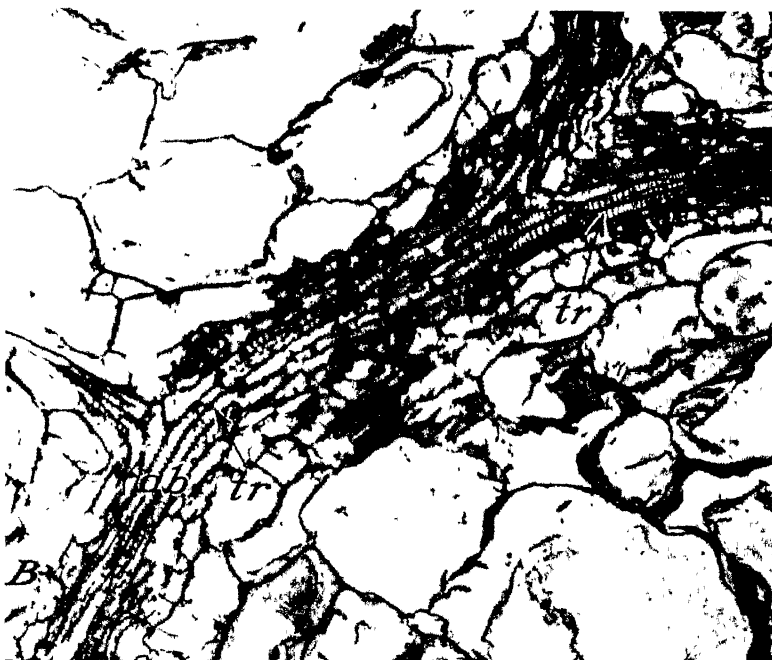
An interesting picture of affected portions of the pericarp and their positions relative to the epidermis and vascular bundles is shown in the transverse section in plate 4, *B*. The epidermis is wavy, being sunken above portions where the underlying parenchyma has collapsed, and unaltered above normal areas and in regions where cavities occur. The dense bands of broken-down cells occur between the outer epidermis and the bundles. In the parts where the epidermis is sunken (pl. 5, *A*), the debris of cell walls and cell contents appears to be forced together; while in the portion where the large cavity appears above the bundle (pl. 5, *B*), fragments of debris have collected around the tracheae and dismembered cell walls are found in the resulting cavity. A similar cavity intermediate between the epidermis and a point near the union of two normal bundles, which are surrounded by unaffected parenchyma, is shown in plate 7, *A*. The accumulation of the dense cellular material between the epidermal layer and the bundles may account for the misconception that the bundles are involved.



*A*, Seemingly enlarged and discolored bundles with connecting veins and associated tissues as they appear in a cleared wall from a blotchy fruit. The small area (*a*) is shown in plate 7, *A*, and the larger central portion (*b*) is shown in *B*.  $\times 10$ . *B*, Transverse section of fruit wall shown in *A*, *b*. Note the distribution of the bundles ( $v_1$ ,  $v_2$ ,  $v_3$ ,  $v_4$ ) and their relation to the broken-down tissues. The large cavity in the center is through the middle of the dark area shown in *A*. It is the result of rupture during removal of the outer epidermis. See plate 7 for details of the marked areas *a* and *b*.  $\times 10$ .



*A*, Transverse section designated in the upper left, (*e*) of plate 6, *A*, taken in the region of the union of the two bundles. The epidermis (*e*) is shown to the upper left, with a large cavity (*c*) and dark tissues between it and the normal bundles (*nb*).  $\times 50$ . *B*, Section from area *a* in plate 6, *B*, between the two small bundles (*nb* and *tr*). The deteriorated cell material between these bundles has not been compressed as in plate 3, *A*.  $\times 33$ . *C*, Large cavity shown at *b* in plate 6, *B*, with a margin of discolored and broken-down cell material. Observed with the naked eye or a hand lens, the whole cavity and margin would appear to be an enlarged bundle. See *D* for details  $\times 33$ . *D*, Lower portion of cavity shown in *C*. Observe the accumulation of cell debris around the large open tracheae (*tr*) which form the rigid framework for the distorted material. Compare with normal bundle (pl. 2, *E*).  $\times 300$ .



4, A, A longitudinal tangential section through the outer wall of a blotchy fruit. Note the broken-down parenchyma (*p*) to either side of the large bundle (*b*) and between the branches of the anastomosing bundle (*ab*).  $\times 9$  B, The anastomosing bundle (*ab*) shown in A where it seems to be necrotic. At this magnification the spiral thickened tracheae (*tr*) appear normal.  $\times 150$ .



*A*, Transverse section through a large bundle with adjacent cavities (*c*). The radiating strands of cell debris appear to have been draped over the strands of tracheary tissue. Note that the parenchyma of the vascular rays found in a normal bundle (pl. 2, *E*) are included in the amassed material.  $\times 250$ . *B*, Longitudinal view of an adjacent portion of the bundle shown in *A*. The large cavity (*c*) is bordered at the left by distorted cell material, which also covers the tracheae (*tr*) to the right of the cavity.  $\times 250$ .

Where small, diffused spots appear between the bundles, a different condition exists than in areas where dense accumulations of cellular material occur. The diffused spots are usually made up of broken-down cells which have not been forced together into a dense mass, but are scattered individually throughout the space they formerly occupied (pl. 7, *B*). This scattered arrangement of dismembered cells almost always occurs midway between two bundles (pls. 6, *B* and 7, *B*) and may be considered as a stage intermediate between the normal and the condition where bands of dense material are formed. The bundles associated with the disintegrated parenchyma have been found to be unaffected in almost every case (pls. 7, *B* and 8, *B*). Longitudinal tangential sections of blotchy fruits show this relationship of the bundles to the broken-down parenchyma quite clearly (pl. 8, *A*).

In severe cases of blotchy ripening, large, dense masses of dead cellular material are found to occur in the bundles in such manner that even the tracheae appear affected (pls. 6, *B*; 7, *C* and *D*; and 9, *A* and *B*). Usually, well-defined cavities are present within the necrotic bundle, and the whole mass may give the appearance of the complete distortion of all the elements of the bundle. Nevertheless, under high power one finds the radiating strands of cell debris have been pulled in around the strands of tracheae, which form the framework on which the amassed material is draped. The cells that are actually affected include, for the greater part, the thin-walled parenchyma of the vascular rays, while the tracheae are uninjured and appear to have functioned normally in the areas beyond the blotch (pls. 8, *B* and 9, *A*).

#### DISCUSSION

The histological observations reported here, as well as data from other investigations of the disorder, substantiate the hypothesis advanced by Seaton<sup>6</sup> (12) that blotchy ripening is due primarily to conditions resulting from the withdrawal of water from the fruit during periods of excessive transpiration occurring 2 to 5 days before the fruit ripens, and indicate that deficiencies of potassium and nitrogen, as suggested by Bewley and White (2), are probably secondary and occasional. It is evident that blotchy ripening is closely associated with the break-down of the parenchyma near and adjacent to the vascular bundles of the maturing ovary walls. That some physical force is operative in bringing about the conditions antecedent to blotchy ripening is also strongly suggested. Moreover, the very location of the affected tissues indicates that a strong pull, exerted through the vascular system upon the normally large, turgid, thin-walled, delicate parenchyma cells between the bundles and connecting veins of the fleshy layer, results in their rupture and distortion. Indeed, areas similar to that shown in plates 4, *B* and 5, *A* suggest a cleavage of tissue such as might result from loss of turgor, the upper portion being supported by the denser tissues of the outer portion of the pericarp, while the portion below lacks such support and is pulled away.

MacDougal (8) has shown by the use of an auxograph that the daily accretion in size of tomato fruits is connected with temperature and

<sup>6</sup> SEATON, H. L. See footnote 5.



water relations. As the temperature of the fruit attached to the plant rose from 12° or 14° to 26° or 28° C., the volume increased to a point where the increased temperature caused an excessive water loss by transpiration which overbalanced the gain by hydration. The midday shrinkage could not be prevented by watering the plants abundantly. His data show that a water deficit may exist in the fruits because of excessive transpiration regardless of the moisture content of the soil. Seaton,<sup>7</sup> using an aniline dye under greenhouse conditions favoring excessive transpiration, has followed the movement of water from the fruits to the transpiring leaves. On bright, warm days in July the dye appeared in the upper leaves in from 1 to 1½ hours after it entered the fruit, while on rainy or cloudy days 4 to 8 hours elapsed before it could be detected in the fruit pedicel.

Similar changes in the direction of the water stream have been advanced as explanations for similar physiological disturbances, such as the blossom-end rot of tomatoes (3), the internal decline in lemons (1), bitter pit of apples (7), the dropping of blossoms in the tomato (9), and the shedding of unopened bolls in cotton (6).

In the development of some fruits, according to Eames and MacDaniels (5), the cells which later form the flesh of the ovary wall are formed during the early development, and development to the mature condition does not involve cell division. The growth consists mostly of the radial enlargement of cells, and in some fruits the formation of intercellular spaces. Thus, in the tomato, ripening involves, histologically, increase in cell size and change in cell shape, the cells becoming turgid with fluid and the walls excessively thin and delicate. According to Sando (11), Rosa (10), and others, during the ripening processes there is an increase in the percentages of moisture, acids, and sugars, and a decrease in solids, nitrogen, starch, pentosans, crude fiber, and ash. Consequently, the parenchymatous cells of the outer fleshy wall, where the greatest increase in size and the changes in chemical composition occur, become more susceptible to injury as maturity is approached. Since the climatic conditions which usually prevail during the ripening period of the spring crop of greenhouse tomatoes are conducive to excessive transpiration, it is logical to expect the greatest losses from blotchy ripening to occur intermittently during that season.

In the red areas of blotchy fruits the normal ripening and coloring are not retarded, and the processes are completed. However, with the break-down of the parenchymatous cells near and adjacent to the bundles in the blotchy areas, the connections which the outlying cells have for the transfer of elaborated foods and water are severed, and they are deprived of the materials which are essential for normal ripening. Consequently, these areas remain hard and develop the colorless or glassy appearance characteristic of the disorder.

#### SUMMARY

A histological study of tissues of greenhouse tomatoes affected by blotchy ripening is reported.

This disorder, which affects greenhouse tomatoes that ripen in May, June, and July, is characterized by a failure of areas of the outer fruit wall to develop and color normally. As the fruit approaches maturity, these areas remain hard and green; and as ripening proceeds further,

<sup>7</sup> SEATON, H. L. See footnote 5

they assume a waxy or glassy appearance. The vascular bundles lying beneath the blotches invariably appear brown and necrotic, and in severe cases cavities may appear adjacent to them. The disorder is restricted to practically mature fruits.

Anatomically, the vascular system of the normal fruit wall consists of large carpellary bundles, their branches, and anastomosing veins. The outer fruit wall is made up of the outer epidermis, the fleshy layer of large parenchyma cells, and the inner epidermis. The bundles are bicollateral, dissected with vascular rays of parenchyma, and with spirally thickened tracheae.

In cleared sections of blotchy fruit walls, large dense areas attendant to the bundles are apparent, while less dense, slightly diffused areas appear between them. Cavities frequently occur in the bundles in severe cases. Histological examination reveals that the discolored tissues are in all cases parenchyma of the fleshy layer and that the bundles are not involved. Bands of discolored cellular material appear between the epidermis and the bundles, which produce the impression that the bundles are affected when viewed through the epidermis. The cavities found in blotchy portions are the result of a collapse of the parenchyma which originally occupied these areas. The diffused spots are composed of dismembered cells from the fleshy layer. Cavities in the bundles result from a collapse of the vascular ray parenchyma which is pulled in around the unaffected tracheae.

The break down of the parenchymatous cells near and adjacent to the bundles in the blotchy areas severs the connections of the outlying cells for a transfer of elaborated materials and water, and normal ripening is inhibited.

The histological observations reported substantiate an earlier hypothesis advanced by the senior author, that blotchy ripening is primarily due to conditions which result from the withdrawal of water from the fruits during periods of excessive transpiration, occurring 2 to 5 days before the fruit ripens.

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# THE PROGRESS OF THE DISTRIBUTION OF SALT IN HAM DURING THE CURING PROCESS<sup>1</sup>

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## INTRODUCTION

Practically all methods of curing pork involve the use of salt as a curing agent. Its main function is as a preservative to prevent or reduce the action of micro-organisms which might cause spoilage in the meat. Aside from this preserving action, the salt contributes greatly to the palatability of the meat. Oversalted pork is undesirable, although it is quite common. The amount of salt present should be sufficient to exert the required preserving action but should not be so excessive as to impair the palatability of the product. In addition the salt should be uniformly distributed throughout the meat to insure uniform quality and palatability.

The process of curing ham involves the application of the curing agent, usually a combination of salt, sugar, and either saltpeter ( $\text{KNO}_3$ ) or potassium nitrite ( $\text{KNO}_2$ ), to the meat by one of two methods, either by dry curing or brine curing. The meat is then allowed to remain in the curing medium for a period of time, which is dependent on the size of the cut and the kind of cure. Subsequently it is soaked in water to remove the excess salt from the outside, smoked, and finally aged or held as long as is desirable to improve its flavor or as is required for practical purposes.

The curing process has as its first objective the penetration of the salt into all parts of the meat rapidly enough to overcome or prevent the deleterious effect of micro-organisms. If the meat is dry-cured an allotted amount of the curing agent is rubbed into it, usually in two or three portions at intervals of several days. Usually the last portion is applied no later than 10 days after the beginning of the cure. In brine curing, which is probably more commonly used, the meat is allowed to remain in a brine or pickle for a period, the length of which should depend on the strength of the pickle used and the temperature at which the cure is conducted. Commercially it is a common practice to pump a strong salt solution into the ham previous to placing it in the pickle proper.

Other things being equal, the effectiveness of salt in preventing bacterial action should depend largely on the temperature at which the curing process is conducted. The optimum temperature is one which is high enough to permit the salt to penetrate as rapidly as possible, and yet low enough to inhibit excessive bacterial action. Temperatures from about 36° to 40° F. seem to fulfill this requirement and are commonly used. Lower temperatures are said to retard the cure,<sup>2</sup> and at higher temperatures spoilage of the meat is liable

<sup>1</sup> Received for publication Sept. 16, 1935; issued March 1936.

<sup>2</sup> MOULTON, C. R. MEAT THROUGH THE MICROSCOPE; APPLICATIONS OF CHEMISTRY AND THE BIOLOGICAL SCIENCES TO SOME PROBLEMS OF THE MEAT-PACKING INDUSTRY. 528 pp., illus. Chicago, 1929. See p. 45.

to occur before a sufficient concentration of salt is obtained to check bacterial action.

The authors have in progress a study of the factors involved in the curing of ham and bacon. The present paper reports their findings in experiments made to determine the degree of distribution of salt in hams cured by different methods, the salt distribution being related to the time allotted to the curing process proper and the time of any subsequent holding or aging.

### EXPERIMENTAL PROCEDURE

Fifty-nine hams weighing from 14 to 19 pounds were used for curing. The plan followed was, whenever possible, to put down together, in the curing agent, both the right and left hams from the same hog, and to remove this pair of hams from cure at the same time, then to soak them in water to remove excess salt or curing agent from the outside. One ham was analyzed immediately before being smoked, and the other was smoked and held for a designated number of days, when it was analyzed also. The latter ham gave

information as to the effect of holding the ham for an additional period and served as a check on the first.

Each ham was sampled by taking a center cut across it next to the aitch bone.<sup>3</sup> This cut was about 1 inch thick, 3 inches wide, and as long as the thickness of the ham. It was divided into five equal horizontal sections which were separately ground in a food chopper.

Each served as a separate

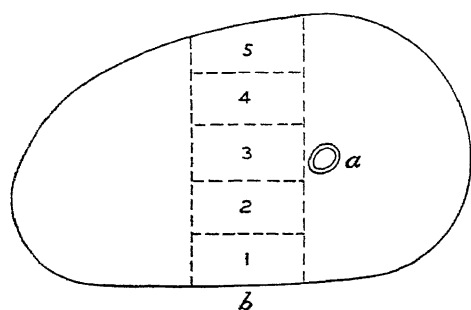


FIGURE 1.—Diagram of method of sampling the hams:  
a, Femur; b, skin side.

sample on which moisture and chlorine were determined. Thus an analysis was obtained on samples representing the center of the ham as well as the two adjacent sections, and the two outside sections. This method of sampling is depicted in figure 1.

The amount of salt present in each section was calculated from the chlorine content as determined by the wet-digestion method as given by Davies.<sup>4</sup> A 5-gram sample of the ground meat was weighed into an Erlenmeyer flask, and to it were added an excess of one-tenth normal silver nitrate, 10 cc of saturated potassium permanganate solution, and 25 cc of concentrated nitric acid. This mixture was boiled vigorously until all solid particles of the meat were completely digested, the average time required being about 20 minutes. Perforated glass beads were used to prevent bumping during the digestion. The solution was then cooled by immersing the flask in cold water. When cool, the excess of silver nitrate was titrated with potassium thiocyanate in the presence of 2 cc of saturated ferric alum and 10 cc

<sup>3</sup> MOULTON, C. R. See p. 112 of reference cited in footnote 2.

<sup>4</sup> DAVIES, W. I. THE DETERMINATION OF CHLORIDES IN DAIRY PRODUCTS AND BIOLOGICAL MATERIAL. Analyst 57: 79-85. 1932.

of acetone. By having a total volume of not more than 100 cc, an easily distinguishable brownish-red end point was obtained without filtering off the white precipitated silver chloride from the yellow solution resulting from the digestion. A blank determination on fresh pork showed it to be so low in chlorine as not to influence the results obtained in this study, in which relatively large quantities of salt were present.

The percentage of salt in the dry matter of each of the five pieces that were subsampled from a ham was computed, and the figures obtained were used to calculate the mean salt content and the standard deviation of the center cut. It should be mentioned that the figures for subsample no. 1 were usually omitted in making the computations, inasmuch as this sample was ordinarily mostly fat and practically always contained less salt than the lean subsamples, presumably due to its lower water content. The coefficient of variability of the salt content was calculated according to the formula

$$cV = \frac{\sigma}{\bar{M}} \times 100$$

in which  $cV$  represents the coefficient of variability.

The value thus obtained is a numerical expression which denotes the extent to which the salt contained in the ham has become distributed or equalized. It is a value which is practically independent of the absolute amount of salt present. Typical figures illustrating the application of this method of expressing the extent of salt equalization are given in table 1, the salt distribution for these two hams being quite different because of different methods of curing.

TABLE 1.—Data obtained from the five subsamples made from center cuts of two hams cured by different methods and indicating the extent to which the salt was distributed throughout<sup>1</sup>

Subsample no.—	Salt in ham no.—	
	1	2
	<i>Percent</i>	<i>Percent</i>
1 (fat side).....	1.05	1.50
2.....	2.35	8.50
3 (middle piece).....	2.89	8.97
4.....	9.87	8.46
5 (flesh side).....	18.89	10.82
Mean salt content of entire center cut.....	7.01	7.65
Coefficient of variability.....	90.9	12.1

<sup>1</sup> All analyses are expressed as percentages of the dry matter.

As illustrated by these two hams, the coefficient of variability is high when the distribution of salt in a ham is not uniform, as in the case of ham no. 1, in which the salt is relatively more concentrated in subsamples 4 and 5. Conversely it will be low when the salt content of the several sections sampled is approximately the same, as in ham no. 2. Thus the coefficient of variability is a useful criterion in judging the extent of the distribution of salt and presents a means of comparing different hams in this respect.

It should be pointed out that the mean salt content of the five pieces subsampled from the center cut is not the true mean salt content of the whole ham. This is apparent when it is recognized that the outer portion of the ham (subsamples 5 and 1) represents, on the basis of weight, a different percentage of the whole ham than it represents in relation to the center cut from which it is taken. The same difference exists in a different proportion for the other subsamples of the center cut. As given in table 1 the mean salt content of the entire center cut is 7.01 percent for ham no. 1 while the salt content as determined for the whole ham after grinding it and subsampling was 5.36 percent. The method of center-cut sampling, therefore, is conventional but serves a useful purpose for comparative studies. In addition it serves as an accurate criterion when a consideration of salt penetration in relation to the thickness of the meat is desired, this aspect being equally as important as the relationships involving the weight of the meat.

In table 2 are given data concerning three different brands of standard, first-quality, packer's ham which are taken to be representative of this type of product. These hams were purchased in a local butcher shop and analyzed in the usual manner.

TABLE 2.—Salt distribution in first-quality commercial hams of three different brands

Part of ham or sample	Salt in ham no —		
	1	2	3
	<i>Percent</i>	<i>Percent</i>	<i>Percent</i>
Middle sample (no. 3).....	7.5	12.9	11.2
Outside sample (no. 5).....	13.9	15.5	18.1
Mean salt content of entire center cut.....	11.6	15.8	14.0
Coefficient of variability for center cut.....	25.8	16.1	24.6

No information is at hand concerning the manner in which these hams were cured or how long they were out of the cure before being sampled. The analysis shows that they contained a considerable amount of salt and that, particularly in the case of hams 1 and 3, the salt content of the outside pieces was somewhat higher than that of the center sample. The coefficients of variability bear out this observation insofar as salt distribution is concerned. A value around 25 would indicate only a fair distribution; thus it is possible that at least two of these hams (nos. 1 and 3) were not held long after curing, or that some other feature of the cure was such as did not permit much equalization of the salt.

#### SALT DISTRIBUTION IN DRY-CURED HAMS

In table 3 are given data for hams cured under experimental conditions. Both groups were dry cured with salt and saltpeter. The hams having the same curing period were from the same hog.

The curing agent was applied at the rate of 1 ounce per pound to the skinback hams and 2 ounces per pound to the regular hams, a portion as large as could be immediately taken up by the meat being applied in one rubbing, the remainder being sprinkled on the meat. The curing was done in wooden boxes from which the liquor exuding from the hams was allowed to drain, and in a refrigerated room where

the temperature was controlled to about 37° F. Pairs of hams were removed from the cure at the same time, one ham from each pair being immediately analyzed, and the other being held for an additional period, as indicated in the table, during which time it was smoked and then analyzed. The hams were held in a refrigerated room previous to smoking, and at room temperature after smoking.

TABLE 3.—*Salt distribution while curing and after holding in hams dry-cured for various periods with salt and saltpeter at 37° F.*

Ham no. <sup>1</sup>	Regular hams			Ham no. <sup>1</sup>	Skinback hams		
	Length of curing period	Length of holding period after cure	Coefficient of variability of salt		Length of curing period	Length of holding period after cure	Coefficient of variability of salt
	<i>Days</i>	<i>Days</i>			<i>Days</i>	<i>Days</i>	
7 <sup>1</sup> .....	20	0	123.8	3 <sup>4</sup> .....	28	0	92.8
1 <sup>2</sup> .....	35	0	100.9	10 <sup>4</sup> .....	35	0	98.5
5 <sup>3</sup> .....	42	0	81.9	2 <sup>4</sup> .....	42	0	76.7
10 <sup>3</sup> .....	20	30	52.8	5 <sup>4</sup> .....	28	32	14.0
2 <sup>3</sup> .....	35	30	25.8	1 <sup>4</sup> .....	35	32	36.0
3 <sup>3</sup> .....	42	30	34.4	6 <sup>4</sup> .....	42	32	20.0

<sup>1</sup> The superior figures are for laboratory records.

The results indicate that with both methods of curing the equalization of salt was progressing throughout the whole period of observation, which was from 20 to 74 days. However, the coefficients of variability are somewhat lower in five out of the six cases for the skinback hams, indicating a somewhat more rapid cure. The slower cure with the regular hams may be attributed to the heavier fat covering on the hams, this layer of fat retarding salt penetration.

It is apparent that the type of results presented in this paper show considerable variation because of the small number of hams that have been tested. Therefore, the results are of limited value in making close comparisons unless large numbers of observations are made, but are useful for the present purpose in indicating the general progress of salt penetration and equalization.

In table 4 are presented data for hams dry-cured with salt, brown sugar, and saltpeter, using 1 ounce per pound of meat, applied in three rubbings. One series of hams was kept during the cure in an outside brick building which was unheated, and with the temperature uncontrolled. The conditions simulated those that would be encountered in farm practice. The other series was cured under controlled conditions.

Salt alone or in combination with saltpeter is commonly used in farm practice for curing meats, but it is generally conceded that a product of better quality is obtained if the curing agent contains some sugar. Unpublished experimental work by the authors has shown that a mixture of 8 pounds of salt, 3 pounds of brown sugar, and 3 ounces of saltpeter is a satisfactory curing mixture.

Data for an odd ham are presented in the controlled series, its mate having been erroneously cut before the termination of the cure; also hams 2<sup>A</sup> and R<sup>2</sup> were not from the same hog but were practically identical otherwise.



TABLE 4.—Salt distribution, while curing and after holding, in hams dry-cured for various periods with salt, brown sugar, and saltpeter, under uncontrolled temperature conditions and at 37° C.

Ham no. <sup>1</sup>	Cured with salt, brown sugar, and saltpeter, curing temperature uncontrolled (40°-60° F.)			Ham no	Cured with salt, brown sugar, and saltpeter at 37° F.		
	Length of curing period	Length of holding period after cure	Coefficient of variability of salt		Length of curing period	Length of holding period after cure	Coefficient of variability of salt
	Days	Days			Days	Days	
7	28	0	67.7	24	21	0	108.8
1	32	0	104.1	34	26	0	111.9
3	42	9	57.3	6	28	0	120.8
10	28	32	18.5	102	33	0	55.3
1	35	32	12.8	7	35	0	70.7
64	42	32	2.9	22	40	0	59.8
				5	42	0	48.7
				R <sup>2</sup>	20	30	32.3
				6 <sup>2</sup>	26	30	12.7
				1	28	30	15.9
				2	35	30	12.2
				14	40	30	8.4
				3	42	30	11.0

<sup>1</sup> The superior numbers and letters indicate skinback hams. The other hams were regular.

In all the hams that were cured for 26 days or more and then held for 30 days, the distribution of salt, as judged by the coefficients of variability, was uniformly good. It appears that salt equalization was progressing at a rather uniform rate during both the curing and the holding periods and would be satisfactory after 50 to 60 days. Also there is some indication that the distribution of salt in these sugar-cured hams, as related to time, is somewhat better than in the case of hams cured with salt and saltpeter under controlled conditions. A comparison of hams that were cured for approximately the same periods and then held for a period after cure, shows that the coefficients of variability are lower in all cases for the sugar-cured hams.

#### SALT DISTRIBUTION IN BRINE-CURED HAMs

The results obtained with two series of brine-cured hams are given in table 5. The brine was made up of 8 pounds of salt, 3 pounds of brown sugar, and 3 ounces of saltpeter, dissolved in 4 gallons of water and used at the rate of 4 gallons per 100 pounds of meat. The series of hams, for which data are given in table 5, were in part cured under controlled temperature conditions at about 37° F., and in part under uncontrolled temperature conditions in the smokehouse previously described.

In the cases where comparisons are possible, it does not appear that there is much difference in salt equalization in the hams cured under either uncontrolled or controlled conditions.

The coefficients of variability of the salt of the hams cured in brine under controlled conditions are about the same for all the hams analyzed after 25 to 42 days in the brine. The figures for hams cured from 25 to 30 days appear to be lower than for those dry-cured for approximately the same period. It might be expected that such might be true since, in dry curing, all the salt which is ultimately

distributed throughout the ham is rubbed into the surface within the first few days after the beginning of the cure. From then on the salt is penetrating further into the ham and is becoming distributed throughout it, the concentration of salt gradually being diminished in the outside portion. In brine curing, salt is being continually taken up by the meat from the brine until the cure in the brine is terminated. It would appear from the coefficients of variability, that in brine-cured hams the concentration of salt in the outside portion in relation to the concentration in the inside is never as great as it is in the early stages of dry curing. Because of the continued adsorption of salt during the brine cure, equalization of the salt may not be as rapid as in dry curing during the cure proper. The data show that, in the case of brine curing, most of the equalization occurs during the holding period following the cure.

TABLE 5.—Salt distribution in hams brine-cured for various periods, both at about 37° F. and under uncontrolled temperature conditions, while curing and after holding

Ham no <sup>1</sup>	Cured at about 37° F			Ham no	Cured under uncontrolled temperature conditions (40°–60° F)		
	Length of curing period	Length of holding period after cure	Coefficient of variability of salt		Length of curing period	Length of holding period after cure	Coefficient of variability of salt
	<i>Days</i>	<i>Days</i>			<i>Days</i>	<i>Days</i>	
1 <sup>B</sup> .....	25	0	84.7	0 <sup>A</sup> .....	28	0	101.3
7.....	28	0	83.5	3 <sup>A</sup> .....	35	0	72.0
51 <sup>E</sup> .....	30	0	81.0	2 <sup>C</sup> .....	35	0	65.3
X <sup>E</sup> .....	34	0	80.5	X <sup>C</sup> .....	35	6	65.1
2.....	35	0	90.9	4 <sup>A</sup> .....	42	0	54.6
5.....	38	0	84.6	1 <sup>C</sup> .....	35	30	31.8
1.....	42	0	73.6	2 <sup>A</sup> .....	28	61	10.6
0 <sup>B</sup> .....	42	6	73.6	1 <sup>A</sup> .....	35	54	9.5
0.....	28	30	3.2	5 <sup>A</sup> .....	42	61	13.1
0 <sup>E</sup> .....	34	30	33.2				
2 <sup>B</sup> .....	35	32	17.1				
3 <sup>B</sup> .....	42	30	13.6				
3.....	35	64	17.8				
6.....	42	60	6.4				

<sup>1</sup> The superior letters are for laboratory records.

No mention has been made of the influence of smoking the meat on the salt penetration. It might be expected that exposure to the higher temperature of the smokehouse would hasten the equalization of salt, but as yet the present study has not particularly concerned this point and such evidence as has been obtained is inconclusive, although there is some indication that smoking slightly speeds up salt equalization.

With respect to the ultimate distribution of salt, the dry-curing and brine-curing processes are essentially the same. However, they must differ in fundamental details. Experiments are cited by Callow <sup>5</sup> in which small pieces of pork muscle were immersed in concentrated solutions of salt. At first, water diffused from the muscle to the solution, the rate of diffusion depending on the concentration of the salt solution. Finally, the water began to diffuse back from the strong solution of salt to the muscle. Apparently this phenomenon

<sup>5</sup> CALLOW, E. H. THEORY OF CURING. [Gt. Brit.] Dept. Sci. and Indus. Research, Food Invest. Bd. Rept. 1932: 101-102. 1933.

does not occur except in the presence of proteins, there being formed a salt-protein complex which has an osmotic pressure greater than that of the surrounding solution.<sup>6</sup> Since the salt-protein complex is hypertonic to the solution, water diffuses back into the protein-containing system.

Callow<sup>7</sup> has also shown that the uptake of salt from solutions of different strengths is continuous and is dependent on the strength of the brine used, more salt being taken up from the stronger solutions.

These findings agree with those of practice, the increase in weight of brine-cured hams due to uptake of water being commonly recognized. The results herein presented show, however, that under ordinary conditions the salt in a ham is not uniformly distributed for some length of time. It is doubtful, with a heterogeneous-phase composition with respect to salt and tissue constituents, whether equilibria with respect to salt and water uptake are reached in ordinary curing practice. Methods of curing, therefore, must be controlled procedures which produce the desired effect.

#### SUMMARY

A statistical method is presented, by means of which it is possible to express mathematically the extent of the distribution of salt in ham at various stages in the process of curing.

Results are given for 59 hams, variously cured.

The salt in hams cured by various methods did not become well distributed throughout the meat until approximately 60 days after the beginning of the various cures. Salt equalization begins in dry-cured hams as soon as the last portion of curing mixture is applied to the meat. In brine curing, salt equalization is accompanied by absorption of salt from the brine until the meat is removed from it, thereafter salt equalization alone takes place.

In all the curing methods used, the aging of hams for 30 days after their removal from the cure permitted further equalization of the salt and gave a product of more uniform salt content.

<sup>6</sup> ADAIR, G. S. A THEORY OF PARTIAL OSMOTIC PRESSURES AND MEMBRANE EQUILIBRIA, WITH SPECIAL REFERENCE TO THE APPLICATION OF DALTON'S LAW TO HÆMOGLOBIN SOLUTIONS IN THE PRESENCE OF SALTS. Roy. Soc [London], Proc A 120: 573-603, illus. 1928.

<sup>7</sup> CALLOW, E. H. SECTION C—PIG PRODUCTS. [Gt. Brit.] Dept Sci and Indus Research, Food Invest. Bd. Rept. 1929: 67-73, illus. 1930. See p 69

# THE EFFECT OF FREEZING ON THE PHYSICAL AND MICROSCOPIC CHARACTER OF GELS OF CORN AND WHEAT STARCHES<sup>1</sup>

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## INTRODUCTION

The number of changes both in the gross and in the microscopic appearance of starch gels brought about by freezing is so great that it suggests many lines of study in connection with the structure of starch granules. It is believed that the facts established in this investigation may be applicable to the problem of low-temperature preservation of food and to that of water removal from food materials containing gelatinized starch. The differences found between corn and wheat starches may be of value in determining the kind of starch for practical use.

## METHODS

### SOURCE AND PREPARATION OF STARCHES

Starches of both corn (*Zea mays*) and wheat (*Triticum vulgare*) were prepared by methods designed to leave them as nearly in their native state as possible, while at the same time freeing them from other constituents of the kernel. Three varieties of corn, Reid Yellow Dent, Krug Yellow, and Champion White Pearl, were used as sources of starch. So little difference between them was found that photomicrographs for the Reid corn only are shown.

The corn was soaked overnight in cold water and ground several times to medium-fine particles. Its starch was washed out and separated from the other material of the kernel, first on a 60-mesh screen, then on fine bolting cloth. The starch suspension was centrifuged and washed in turn with 2-percent sodium chloride, water, alcohol, and ether. Layers of gluten at the top of the starch layer in the centrifuge bottles were scraped off. This starch was air-dried for use.

Wheat starch was prepared by the method previously reported by Woodruff and Webber (9)<sup>2</sup> from 1934 Fulvio wheat flour milled especially by a commercial soft-wheat mill for another experiment station project. Slight physical differences between starches separated from bleached and unbleached flours were found, but microscopic differences were negligible and photomicrographs only of unbleached flour starches are shown.

### FREEZING OF STARCH GELS

Starch gels were made by heating suspensions containing 5 percent of starch by weight in a boiling water bath. The starch was first moistened with a little cold water in a conical flask. The boiling water was added and the flask swirled until a thermometer in the

<sup>1</sup> Received for publication Aug. 5, 1935; issued March, 1936. A brief paper covering part of these findings was read before the food and agricultural division of the American Chemical Society in New York City in April 1935.

<sup>2</sup> Reference is made by number (italic) to Literature Cited, p. 237.

paste reached the temperatures 70°, 75°, 80°, 85°, 90°, and 95° C. The pastes were poured immediately into molds and allowed to cool and set to a gel overnight. One set of molds was placed in the freezing unit of an electric refrigerator where the lowest temperature reached by them was -2° to -3°. After 5 or more hours they were removed from the freezer, allowed to thaw at room temperature, and examined microscopically. Another set of molds was packed in solid carbon dioxide and left for 1 hour, after which they were removed and allowed to thaw at room temperature. Samples were also frozen in liquid air but these were not different from the ones frozen in solid carbon dioxide.

#### MICROSCOPIC EXAMINATION

Specimens were removed from the gels with a knife tip, special care being taken to avoid cutting the shreds in the frozen ones. The specimens were then mounted in water for microscopic examination. Photomicrographs were made with ordinary light at a magnification of 900 times. Polarized light was also used for the examination of each specimen between crossed nicols.

### RESULTS

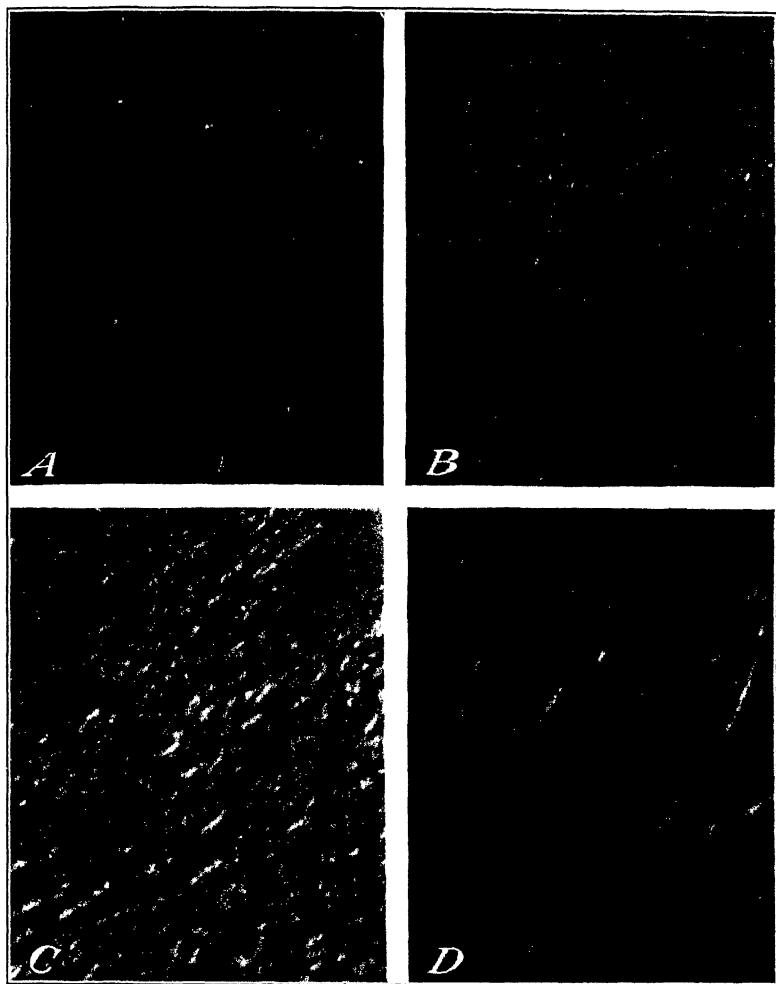
#### PHYSICAL CHANGES IN GELS CAUSED BY FREEZING

The following discussion is for the most part confined to the behavior of gels made by heating starch suspensions to 95° C. The cornstarch made a well-formed gel even at 75° or 80°, but wheat starch did not until it was heated to a temperature of 95°, for at the lower temperatures some starch granules remained unswollen.

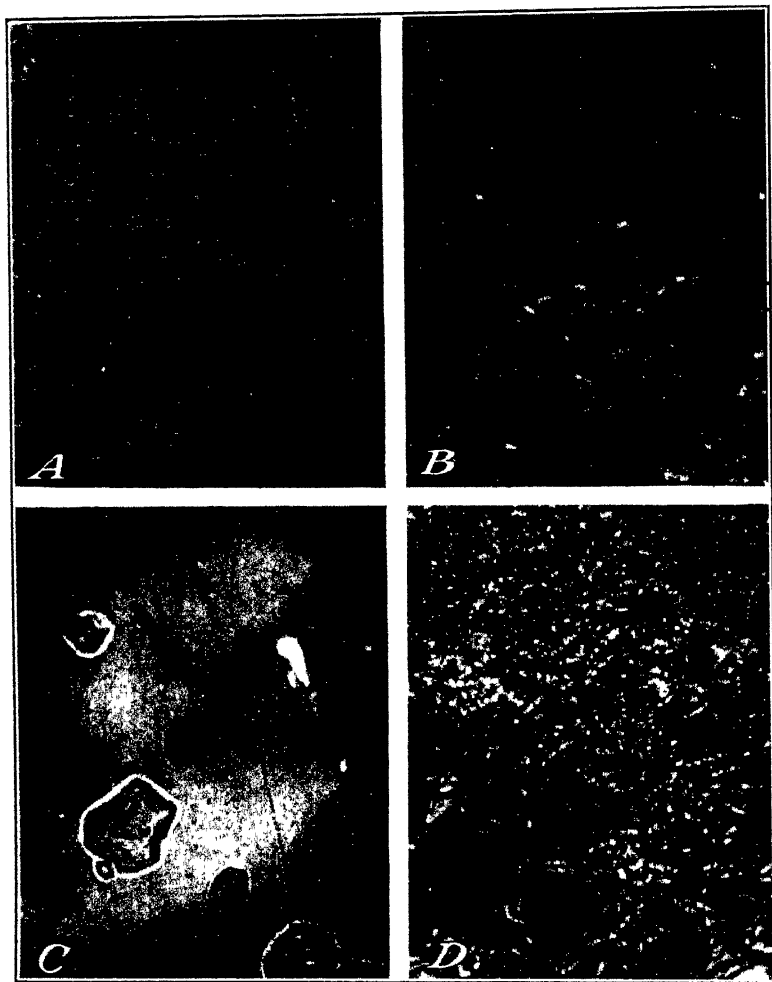
The cornstarch gel after being frozen slowly where it reached a temperature no lower than -2° to -3° C. became coarsely fibrous with some radial orientation of the fibers. It was spongelike in that water could be pressed from it without its losing shape and it greedily absorbed water again. It likewise absorbed water solutions of either iodine or of dyes. The original gel, on the other hand, was not penetrated by an iodine solution dropped on its surface; moreover, it was bluish white, pasty, uniform in consistency so far as the unaided eye could detect, and did not give up water when pressed. It dried in air to a horny film which could be remoistened only with difficulty. The sponge of the frozen gel, pressed free from water, dried in air, giving a porous, fibrous, friable substance which absorbed water as easily when dry as it had when moist. The starch thus retrograded by freezing could be warmed with water and dispersed sufficiently to make a gel again similar in appearance to the original one, though less uniform in consistency. The freezing and dispersing were repeated many times without noticeable loss of power to form a gel. Even in gelatinized suspensions of 1-percent concentration instead of the 5 percent used, the fibrous arrangement could be seen.

Frozen in solid carbon dioxide, the gel changed in gross appearance much less; it retained most of its pasty appearance and though it gave way when pressed, no water came from it. It dried to a horny film very like the original gel.

Wheat-starch gels frozen in the refrigerator were fibrous like those of cornstarch, but the sponge formed was not so tough and water could not be squeezed out so readily. They differed decidedly from



Photomicrographs of frozen cornstarch gels. *A*, Gel before freezing; *B*, 1-percent suspension, frozen at  $-2^{\circ}$  to  $-3^{\circ}$  C.; *C*, gel frozen in solid carbon dioxide; *D*, gel frozen at  $-2^{\circ}$  to  $-3^{\circ}$ . All were gelatinized at  $95^{\circ}$ ; all except *B* contained 5 percent starch.  $\times 900$ .



Photomicrographs of frozen starch gels and of optically active particles in starch. *A*, Wheat-starch gel before freezing; *B*, wheat-starch gel frozen at  $-2^{\circ}$  to  $-3^{\circ}$  C; *C*, cornstarch gelatinized at  $75^{\circ}$ ; *D*, wheat starch gelatinized at  $85^{\circ}$  and then frozen in solid carbon dioxide. Note optically active particles in *C* and *D*.  $\times 900$ .

the frozen cornstarch gels in that they dried to a horny mass which did not readily reabsorb water. This point was noted by Holton,<sup>3</sup> who worked on a similar problem in this laboratory. Gels frozen in solid carbon dioxide were again more like the original ones and lacked the fibrous structure produced at  $-2^{\circ}$  to  $-3^{\circ}$  C.

#### MICROSCOPIC APPEARANCE OF FROZEN GELS

Freezing effected changes as pronounced in the microscopic appearance of gels as in the gross. Evidence of these changes is presented in plates 1 and 2.

It was not easy to show the structure of a starch gel microscopically. The swollen granules were very transparent after being heated to  $95^{\circ}$  C., were packed against each other, and their details were indistinct (pl. 1, *A*). The remarkable change in appearance brought about in cornstarch gels by freezing is shown in plate 1, *C* and *D*, which presents photomicrographs of a gel frozen in solid carbon dioxide and of one placed in the freezing unit of a refrigerator where it reached a temperature of only  $-2^{\circ}$  to  $-3^{\circ}$ . The very heavy veins in the latter seemed to correspond to the fibrous structure noticed on gross examination. Freezing more quickly at the lower temperature produced less change in microscopic as well as in gross appearance, though veining of a light nature was apparent. Plate 1, *B*, shows that even in a 1-percent suspension, gelatinized starch was profoundly altered by freezing; long fibers are apparent there.

Wheat starch became even more indistinct in outline than cornstarch when gelatinized at a temperature of  $95^{\circ}$  C. Plate 2, *A* and *B*, shows the wheat starch as it appeared in the original gel and after being frozen at  $-2^{\circ}$  to  $-3^{\circ}$ . Definite lines of reorientation are visible in the latter though the heavy veins of frozen cornstarch gel are absent.

Starch grains lost their anisotropy as soon as they swelled slightly. Very small, oval-round particles which transmitted polarized light could be seen, however, after birefringence had disappeared. These particles were somewhat visible at all temperatures of gelatinization though they were more plentiful and more distinct at temperatures under  $95^{\circ}$  C. In many cases where particles occurred singly they showed a Brownian movement. A few such particles can be seen in the cornstarch gel previously heated to  $95^{\circ}$ , shown in plate 1, *A*, and a suggestion of their presence is to be noted also in the wheat-starch gel gelatinized also at  $95^{\circ}$ , of plate 2, *A*, though in the latter case the camera was focused on the outer boundaries of starch granules, hence these small particles were not in focus. Plate 2, *C* and *D*, shows these oval-round particles in corn and wheat starches which had been heated to temperatures of only  $75^{\circ}$  and  $85^{\circ}$  respectively. When slight pressure was exerted on the cover glass during the examination of starch gelatinized at temperatures of  $70^{\circ}$  or somewhat above, such streams of these particles as are shown in plate 2, *C*, were often observed. Short chains of them under or on the surface of the swollen starch granules were noted more frequently than the long streamers just mentioned. The wheat starch of plate 2, *D*, shows a surface on which such particles are visible in great numbers.

<sup>3</sup> HOLTON, M. THE EFFECT OF RETROGRADATION ON THE SUBSEQUENT GELATION OF WHEAT STARCH. Unpublished master's thesis. Univ. Ill. 1934



The veinlike structure appearing in the photomicrographs of frozen gels, particularly of those frozen at  $-2^{\circ}$  to  $-3^{\circ}$  C., also transmitted polarized light. Likewise did dried shreds of frozen starch gels mounted without water (not shown in the plates).

#### DISCUSSION

Only a few investigators seem to have mentioned the appearance of frozen starch gels and no photomicrographs of them have come to the writers' attention. Samec (8) dialyzed gelatinized and frozen potato starch for soluble starch on which he made determinations of phosphorus. Baldwin (1) used freezing as a means of separating alpha and beta amyloses of potato starch. Reilly, O'Donovan, and Murphy (7) centrifuged the clear liquid from the fibers formed by freezing a 5-percent suspension of gelatinized potato starch and recovered an amylose on which to make molecular weight determinations.

The literature contains several references to what might correspond to the oval-round particles pointed out in the accompanying plates. Several investigators, Samec (8), Hanson and Katz (3), and Hess and Rabinowitsch (4), in recent years have discussed crystallin or micellar structure in line with the very early conceptions of starch constitution. Hanson and Katz reproduced with drawings the arrangement of blocklike units in potato, arrowroot, and wheat starches, made suitable for observation by swelling with calcium nitrate. Hess and Rabinowitsch photographed by cinematograph the material issuing from a punctured potato-starch granule. The irregular mass consisted of particles having a Brownian movement. Linsbauer (6) has followed the gelatinization changes in potato starch with a hot-stage microscope; his drawings, however, do not include such particles as are shown in this paper. There is at least a suggestion in the resemblance in appearance of the particles of the accompanying photomicrographs to the cellulose particles found by Farr and Eckerson (2) in cotton fibers, that the particles seen here in starch have something fundamental to do with the structure of the starch granule.

The observation that gels showed a greater change in both gross and microscopic appearance at  $-2^{\circ}$  to  $-3^{\circ}$  C. than they did at much lower temperatures, was in accord with a result of Katz' (5) work on bread staling. The staling process which is concerned with changes in starch was found by him to pass through a maximum value at  $-2^{\circ}$  to  $-3^{\circ}$ , when bread was stored at temperatures varying over a wide range.

A study of the photomicrographs made in the course of this investigation lead the writers to believe that the reticulation of the starch gels, frozen at  $-2^{\circ}$  to  $-3^{\circ}$  C. and later thawed, may possibly have resulted from the association of micelles or aggregates which in turn had formed when the dehydration of the swollen starch granules by ice-crystal formation permitted the molecules to be drawn closer together through secondary valence forces. The explanation offered for lesser change at the still lower temperatures of solid carbon dioxide and liquid air is that less injury occurred with rapid formation of smaller ice crystals, and the temperature was, moreover, too low easily to permit of the physical changes of reorientation. Indications of this lie in the fact that if a gel, previously frozen in solid carbon

dioxide, was kept for many hours in the freezing unit of the electric refrigerator while its temperature rose it would assume practically the same microscopic characteristics as the gel that had been all the time in the unit at  $-2^{\circ}$  to  $-3^{\circ}$ .

#### SUMMARY

Corn and wheat starches were gelatinized at temperatures ranging from  $70^{\circ}$  to  $95^{\circ}$  C. and gels of 5 percent concentration were then frozen at  $-2^{\circ}$  to  $-3^{\circ}$  and also at the temperature of solid carbon dioxide. After thawing they were examined for physical and microscopic changes. In gross appearance the frozen gels were very different from the original ones. At  $-2^{\circ}$ , where the effect of freezing was greatest, the gel became like a fibrous sponge from which most of the water could be pressed. Other changes in physical properties also were noted.

Photomicrographs are shown of corn and wheat starches which had been first gelatinized, then frozen. Veined areas appeared in gels after slow freezing at  $-2^{\circ}$  to  $-3^{\circ}$  C. Rapid freezing in solid carbon dioxide produced fewer changes both in the gross and in the microscopic appearance of gels than did slow freezing at  $-2^{\circ}$  to  $-3^{\circ}$ . The veinings in the frozen gels were optically anisotropic as were also small oval-round particles seen constantly at different stages of swelling.

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## WATER SOAKING OF LEAVES IN RELATION TO DEVELOPMENT OF THE WILDFIRE DISEASE OF TOBACCO<sup>1</sup>

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### INTRODUCTION

Leaf spot diseases, popularly referred to as red rust, black rust, and white rust, have long been a problem in the culture of tobacco (*Nicotiana tabacum* L.), but until recently nothing was known as to either their cause or prevention. During the period 1917 to 1925, however, serious outbreaks of these leaf-spot diseases led to scientific investigations, and in consequence the two most important types of leaf spot were described respectively as wildfire (*Bacterium tabacum*)<sup>2</sup> by Wolf and Foster (16)<sup>3</sup> and blackfire or angular leaf spot (*Bact. angulatum*) by Fromme and Murray (9). Seed treatment, seedbed sanitation, and, in some localities, seedbed spraying were recommended as control measures. These proved very effective in eliminating or greatly reducing the amount of seedbed infection, but they have not been effective in preventing destructive field outbreaks of leaf spot.

The field epidemics occur late in the season and have been closely associated with certain weather conditions and fertilization and cultural practices. Recently there has been a growing tendency to question whether either *Bacterium tabacum* or *Bact. angulatum* is capable of causing destructive leaf spot epidemics in the field, and to regard this leaf break-down as, at least in part, a nonparasitic trouble due to unbalanced nutritional conditions, possibly assisted by over maturity of the leaf. In support of this view Valteau (14) has cited the fact that *Bact. angulatum* when inoculated into leaves produces only very small lesions of a harmless nature, while field blackfire is characterized by large rapidly developing lesions that cause great destruction. In the earlier work, the fact that the organism could be isolated from the large field lesions and would produce small lesions from controlled inoculations led to the assumption that it would also be able to produce the large type of lesion if suitable conditions were provided.

Exactly the same situation exists with respect to *Bacterium tabacum* and wildfire. Artificial inoculations with the organism have always produced lesions consisting of a small central dead area surrounded

<sup>1</sup> Received for publication Sept. 10, 1935, issued March 1936.

<sup>2</sup> Synonym, *Phytophthora tabaca* (Wolf and Foster) Bergey et al.

<sup>3</sup> Reference is made by number (italic) to Literature Cited, p. 288.

by a broad yellow halo (fig. 1, *A*), and these lesions require about a week to develop fully. In the field, on the other hand, lesions of large size may appear in 2 or 3 days with little or no suggestion of a halo (fig. 1, *B*). The halo-type lesions produced by artificial inocula-

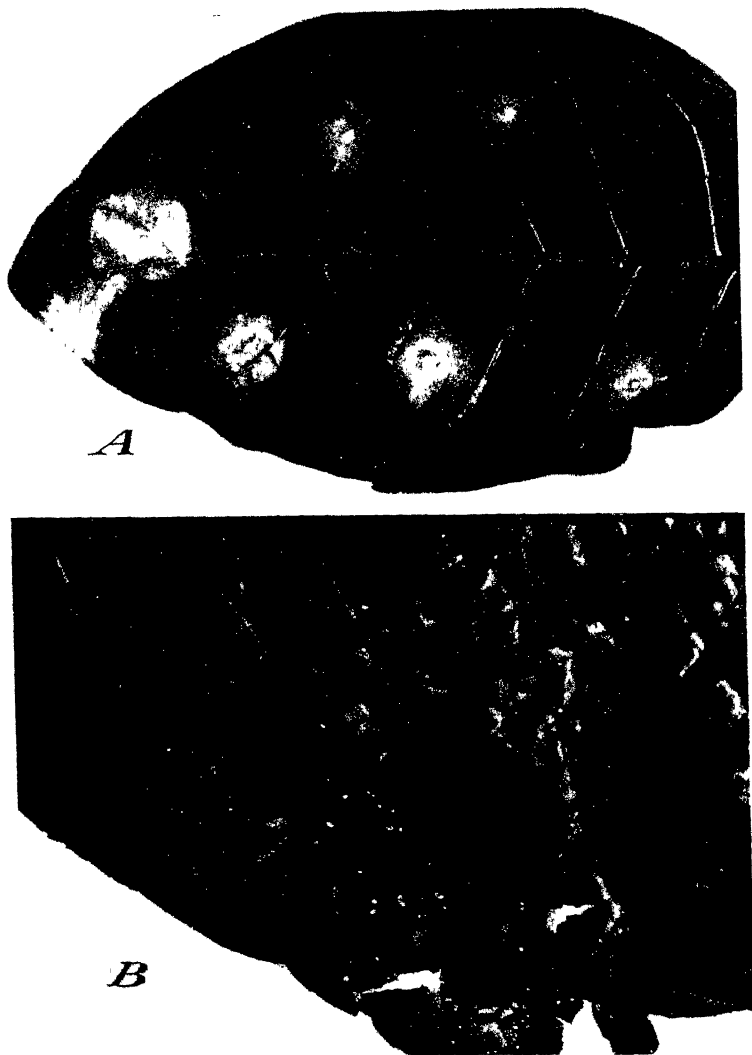


FIGURE 1—*A*, Halo wildfire. Lesions of this type result when young leaves are pricked with a needle dipped in a *Bacterium tabacum* culture. Note the small central dead area surrounded by a broad yellow halo. *B*, Epidemic wildfire. These lesions are typical of those which developed in the field during the leaf spot epidemic of 1933. Note the large irregular dead areas with little or no evidence of halos around the margins.

tion have been regarded as typical wildfire, but the exact cause of the large field-type lesions, referred to in this paper as "epidemic wildfire", has been questioned by various writers. Thus as early as 1921 Chap-

man and Anderson (4) reported that in the Connecticut Valley they found much "rust", which they suggested was probably caused by excess of nitrogen and deficiency of potash. Reference to a non-parasitic rust which might be confused with wildfire is also made by Johnson and Fracker (11). Beach (1) has logically connected the nonparasitic blackfire suggested by Valleau (14) with the nonparasitic rust mentioned above, and he states:

Nonparasitic spots, including what the farmers have called "rust" but referred to by pathologists as "blackfire", often develop on the tobacco at maturity when the weather is very wet.

Thus, both in the southern tobacco region with *Bacterium angulatum* and in the northern region with *Bact. tabacum*, the leaf spot situation is complicated by the possibility that the destructive late-season epidemics are in part nonparasitic. This view is supported indirectly by the failure of recommended control measures to give protection under field conditions, and directly by the fact that artificial inoculations with either organism produce small harmless lesions (fig. 1, A) which fail to develop into anything resembling the large field lesions (fig. 1, B). No one, however, has been able to reproduce the large lesions under controlled conditions by either pathological or physiological methods.

In seeking an explanation of this situation, the writer has conducted work simultaneously with the bacteria mentioned and also with various fungi. The present paper, however, records only the results secured with *Bacterium tabacum*.

The fundamental importance of this study lies in the fact that if the destructive, late-season type of leaf spot is not caused by an organism, then obviously the present control methods, which are directed at eliminating the organism, have no chance of success, and the entire problem of leaf spot control must be reconsidered. On the other hand, if the organism can be proved to be an essential factor in leaf spot development, then the failure of the present control program can be safely ascribed to inadequacy of measures recommended and not to misdirection of efforts. Obviously, the only way to prove definitely either the parasitic or the nonparasitic theory of the cause of leaf spot is actually to reproduce the large destructive type of field lesions under controlled conditions.

#### METHODS OF STUDY

The organism was isolated from material obtained in many localities and from lesions of widely varying appearance. Cultural and inoculation studies with these isolations showed no differences. The pathogenicity of all isolations decreased rapidly in artificial culture, but was readily maintained by frequent inoculation and reisolation. Inoculations were usually made with 6- to 10-day-old broth cultures diluted 10 times with water. Wound inoculations were made with sharp-pointed glass tubes, leaving a small drop of the inoculum at each prick. Following inoculation the plants were incubated in a saturated atmosphere at a favorable temperature (70°-80° F.) for 48 to 72 hours. In prior studies the general practice had been to use very young plants, but in this work large plants were employed for both greenhouse and field studies. Since the different leaves of large plants do not respond in the same manner to infection, it was found advisable to classify

leaves as basal, middle, or tip. All measurements of lesion size refer only to the area of tissue actually dead. Nutrition studies in the greenhouse were conducted with field soils of low fertility to which the various nutrient salts were added in solution at weekly intervals.

Field studies were conducted at the Arlington Experiment Farm, near Rosslyn, Va., and at Upper Marlboro, Md. At the former location the soil was a medium sandy loam of moderate fertility, and at the latter location the soil was a light sandy loam of low fertility. At Upper Marlboro there was available an extensive series of agronomic experiments which provided a wide range of nutritional and topping conditions. The disease was well distributed each year in these plantings, and the epidemic type of wildfire was general in 1933. The Upper Marlboro plantings provided an exceptional opportunity to study the influence of cultural and nutritional factors on disease development. The object of the work necessitated taking data in much detail, often as counts of the number of lesions and measurements of the size of lesions, leaf by leaf. Presentation of any considerable amount of these data is impracticable, and consequently in this report significant developments are summarized and supplemented with representative data. The large volume of negative results secured during the early phases of the study will first be referred to very briefly.

The Maryland Broadleaf variety was used throughout this work, and supplementary tests were conducted with Connecticut Havana and Cash, the latter being a strain of Orinoco.

#### RESULTS OF PRELIMINARY EXPERIMENTS

It has been suggested that the epidemic type of wildfire is due (1) to intensive dissemination of the organism by rain spattering plus high humidity, and (2) to excessive plant susceptibility induced by low topping and high-nitrogen or low-potash fertilization. During the first year and a half of this study, numerous experiments were conducted along these lines.

Plants in the field were sprayed with bacteria during the progress of rains to find whether intensive inoculation under supposedly favorable natural conditions would be effective. In the greenhouse, plants were similarly inoculated and incubated in a saturated atmosphere for various periods and at different temperatures. Excellent infection, as judged by the number of lesions, was obtained in both field and greenhouse experiments. This was favored by temperatures between 70° and 80° F. and by incubation periods of 48 to 72 hours. The lesions that resulted, however, were all of the halo type, and consequently they bore no resemblance to epidemic wildfire.

In other experiments, the effect of height of topping was studied, and it was found that low topping increased both the number and the size of lesions, but again all lesions were of the halo type. Many infection experiments were conducted with leaves of different ages, with inoculations of both the upper and the lower leaf surfaces, both with and without wounding. Wound inoculations were positive under all circumstances. Without wounding, young leaves and lower surfaces were more easily infected than were old leaves and upper surfaces. The lesions secured were all of the halo type, and the results

tended to indicate that halo and epidemic wildfire are different, for in the field old leaves, and not young ones, were most severely injured.

Extensive nutrition experiments were conducted with nitrogen, phosphorus, potassium, magnesium, and chlorine. The effect of both deficiencies and excesses of these elements alone and in combination was considered. High nitrogen consistently increased the number and size of lesions. High potash was more variable in its effect, in some experiments distinctly reducing the amount of infection and in others yielding negative results. Phosphorus and magnesium had little effect on disease development. Chlorine in excess distinctly reduced the amount of disease in some experiments. However, none of these treatments altered the character of the lesions, all of which were of the halo type of wildfire and consequently caused but little actual leaf damage.

The one conclusion from these experiments was that epidemic wildfire had not been produced by any of the methods tried. On the other hand, the failure to produce extensive leaf break-down could not be interpreted as proving the nonparasitic leaf spot conception. Rather, it appeared that the essential cause of epidemic wildfire might have been missed entirely, and later developments proved this to be the case.

The first clue as to the real predisposing factor was obtained during the summer of 1933, when it was possible for the first time to follow the entire development of an epidemic of leaf spot that was associated with a severe wind and rain storm. The storm occurred in August of that year.

#### RELATION OF STORM-INDUCED WATER SOAKING OF LEAVES TO EPIDEMIC WILDFIRE

##### FIELD OBSERVATIONS

During the storm of August 1933 numerous water-soaked areas scattered over the leaves were observed. As the strong wind and heavy rain continued, these areas increased greatly in number and size. Plants were partly tilted over by the wind, and the leaves so turned up became extensively water-soaked (fig. 2). There were noticeable differences in the amount of water soaking, depending on methods of topping and fertilization. These water-soaked areas were plainly the result of flooding of the intercellular spaces, and, following the first violent phase of the storm, they persisted for some 48 hours, until the weather began to clear. As the sun came out, many of these areas cleared up completely, but at the same time many others began to turn brown and die (figs. 3 and 4). Microscopic examinations showed abundant evidences of bacteria in the dying tissues. Isolations yielded practically pure cultures of *Bacterium tabacum*. The lesions that resulted were of the typical epidemic type of wildfire, and the disease caused great destruction throughout the tobacco sections of Maryland and Pennsylvania. As a check on the question of whether the storm itself produced this destructive leaf spot in the absence of the organism, the writer fortunately had a disease-free plot of tobacco at College Park, Md. This plot developed no leaf spot following the storm, regardless of fertilizer or topping treatment.





FIGURE 2.—Water soaking as it developed on leaves in the field following a severe wind and rain storm.



FIGURE 3.—Epidemic wildfire as it appeared on leaves a few days after they had been water-soaked as shown in figure 2. Note the irregular shape of the lesions and the absence of any halo effect. These lesions were red brown and typical of the condition often referred to as rust.



FIGURE 4.—Epidemic wildfire showing dark, zonate lesions of the type often referred to as blackfire. The color, however, is due to the fact that this was a heavy, mature leaf as compared with the immature leaf shown in figure 3. The zonate markings in this case are the indications of smaller wildfire lesions formed at an earlier date, which spread rapidly after a storm and coalesced to form the large dead area shown.

## ARTIFICIAL WATER SOAKING OF LEAVES

As a result of the observations just described, the entire problem of epidemic wildfire was reconsidered. Water soaking had never been encountered in previous inoculation experiments, because the plants had always been dampened with a fine mist. Tests now showed that typical leaf water soaking could be produced with a strong water spray. By using a coarse spray and holding the nozzle about 3 feet away, it was possible to produce large water-soaked areas on leaves laid against a padded board in  $\frac{1}{2}$  to 2 minutes. These treated leaves recovered without injury when they were not inoculated.

## INOCULATION OF WATER-SOAKED LEAVES

Inoculations made in the usual manner, except that the leaves were previously water-soaked, yielded outstanding results. Figure 5 illustrates the disease development in an experiment in which halves of leaves were water-sprayed from above until scattered areas appeared, and the entire leaves were then atomized from below with a suspension of bacteria. The tissues that were water-soaked began to break down after 64 hours, when the plants were removed from the damp chamber, and large dead areas were soon formed which coalesced to produce the dead area shown. This was still enlarging when the photograph was taken, 9 days after inoculation. Obviously, although the water-soaked areas provided the starting points, the ultimate lesions extended far beyond the borders of the original water-soaked areas.

This same sequence also occurs under natural conditions; (1) the quick break-down of the infected, water-soaked areas, which takes place during the few hours following the storm, and (2) the more gradual extension of the lesions, which continues for as much as 2 weeks. In contrast to this, the small halo lesions, on the side of the leaf that was not water-soaked (fig. 5), were not visible until about 5 days after inoculation, and after a few days they ceased to enlarge. It seems evident that the secondary extension of lesions originating with water-soaked areas can be attributed to the mass action of the tremendous population of bacteria within the host tissues. Isolations show that, whereas without water soaking the bacteria remain confined to the tissues close to the point of inoculation, with water soaking large areas of tissue may be swarming with bacteria in as short a time as 30 hours.

Since the major effect of water soaking was to increase the size of the lesions, it seemed desirable to measure this effect quantitatively. For this purpose 25 leaves were selected on plants of different ages, and one-half of each leaf was water-sprayed. Four water-soaked areas were selected on each treated half leaf and inoculated by pricking lightly in the center with a needle dipped in a suspension of bacteria. The half of each leaf not water-soaked was then inoculated similarly, and all were incubated as usual. Each experimental leaf was then outlined on a sheet of paper, and the location of the inoculations and the size of the water-soaked areas were indicated. Outlines were made of all lesions at 3, 7, and 16 days after inoculation. The results from all 25 leaves were similar, and are indicated by the detailed data from 3 representative leaves given in table 1.



FIGURE 5.—Effect of water soaking on disease development. The entire leaf was inoculated but only the right half was water-sprayed before inoculation. The lesions on the right half soon spread and coalesced, while those on the left remained small.

TABLE 1.—Results from *Bacterium tabacum* inoculations on leaf with and without water soaking

Age of leaf	Lesion no.	Size of lesions after indicated number of days					
		Water-soaked and inoculated			Inoculated only		
		3 days	7 days	16 days	3 days	7 days	16 days
		<i>Sq. in.</i>	<i>Sq. in.</i>	<i>Sq. in.</i>	<i>Sq. in.</i>	<i>Sq. in.</i>	<i>Sq. in.</i>
Young.....	1	0.12	0.45	1.58	0	0.06	0.06
	2	.06	.34		0	( <sup>2</sup> )	( <sup>2</sup> )
	3	.09	.90		0	.02	.02
	4	.13			0	.05	.05
Half grown.....	1	.06	.52	1.25	0	.06	.06
	2	.09	.75	2.18	0	.03	.03
	3	.09	.75	2.19	0	.04	.04
	4	.04	.18	1.00	0	.03	.03
Mature.....	1	.02	.30	2.20	0	( <sup>2</sup> )	( <sup>2</sup> )
	2	.04	.09	1.36	0	( <sup>2</sup> )	( <sup>2</sup> )
	3	.08	.50	2.24	0	.03	.03
	4	.08	.75	2.00	0	( <sup>2</sup> )	( <sup>2</sup> )
Total area killed.....		90	5.53	20.22	0	.32	.32

<sup>1</sup> The 4 original lesions coalesced to form 1.<sup>2</sup> Trace.

The figures of table 1 show clearly how it is possible with the aid of leaf water soaking to produce typical epidemic wildfire. Note that with water soaking lesions of appreciable size had formed 3 days after inoculation, while without water soaking no development was apparent at this time. After 7 days, 100 percent of infection had resulted from all inoculations, but the total leaf area destroyed was increased by water soaking 1,628 percent. After 7 days there was no further increase in the size of the lesions on the half leaves not water-soaked, but the lesions with water soaking continued to enlarge, and at the end of 16 days the increase in area killed on water-soaked leaves was 6,218 percent greater than that on leaves not water-soaked. The lesions without water soaking were of the typical harmless halo type, and with water soaking they were of the quick-forming destructive epidemic type. It is to be observed also that with water soaking leaves of all ages were highly susceptible, as indicated by the large size of lesions formed. Without water soaking the mature leaves were most resistant, though the small size of all lesions indicates a high degree of resistance for the normal leaf at any age.

#### EFFECT OF SIZE AND PERSISTENCE OF WATER-SOAKED AREAS ON LESION DEVELOPMENT

Under natural conditions it has been observed that the water-soaked condition of many areas disappears in a few hours, and that when this occurs epidemic wildfire does not result. Both during the extensive storm of 1933 and during a more local but equally severe one in 1934, the water-soaked condition of the areas persisted for 48 hours, and then, as the weather cleared, the break-down of tissues took place. Poured-plate isolations showed that after 15 hours the bacteria were well distributed throughout some of the water-soaked areas but were not numerous. Similar isolations after 30 hours showed an enormous multiplication in numbers. It seemed desirable to find out definitely whether large water-soaked areas tended to facilitate the formation of large lesions. The large areas persist

longer, as a rule, than the small ones, but the effect of persistence will be considered separately. In seeking to evaluate the direct effect of size of water-soaked areas, it was first shown conclusively that if large and small areas are generally inoculated by atomizing the entire leaf surface with a bacterial suspension the lesions resulting from the larger areas are much the more extensive, owing to multiple infections. It is probable, however, that under many field conditions the supply of bacteria is limited, and hence comparisons were made with large and small water-soaked areas inoculated at only a single central point. In these experiments the large water-soaked areas ranged from  $\frac{3}{4}$  to  $1\frac{1}{2}$  inches in diameter and the small areas from  $\frac{1}{4}$  to  $\frac{1}{2}$  inch. All inoculations were incubated 48 hours, and the lesions were measured 5 days after inoculation. The results are shown in table 2.

TABLE 2.—Size of lesions in relation to size of water-soaked areas

Experiment no	Size of lesions on leaves with—					
	Large water-soaked areas			Small water-soaked areas		
	Tip leaves	Middle leaves	Basal leaves	Tip leaves	Middle leaves	Basal leaves
	Sq in	Sq in	Sq in	Sq in.	Sq in	Sq in
1.....	1 1	1 25	1 25	0 25	0 25	1 08
2.....	1 08	1 25	1 56	.29	1 04	1 16
3.....	1 75	1 17	1 22	30	96	.92

Records of the check inoculations, without water soaking, are not included in table 2, because at the time data were taken they appeared merely as faint halos and no dead tissue was observed. The tendency of the large water-soaked areas to facilitate the formation of large lesions is quite apparent, and the data again point to the marked effect of the intercellular liquid in facilitating spread of the bacteria through the tissues.

The relation of persistence of the water-soaked areas to the development of epidemic wildfire was studied by water soaking leaves and then holding the plants for various periods in a saturated atmosphere. The water soaking disappeared as soon as the plants were removed to ordinary greenhouse conditions. The results, taken in terms of percentage of leaf area killed 11 days after inoculation, are presented in table 3.

TABLE 3.—Relation of persistence of water-soaked areas to disease development

Leaf no.	Percentage of leaf killed after 11 days when water-soaked areas were held for—			Leaf no.	Percentage of leaf killed after 11 days when water-soaked areas were held for—		
	10 hours	24 hours	50 hours		10 hours	24 hours	50 hours
	Percent	Percent	Percent		Percent	Percent	Percent
1.....	20	40	35	8.....	15	50	80
2.....	50	30	35	9.....	40	75	75
3.....	20	10	35	10.....	15	75	85
4.....	10	30	30	11.....	60	80	95
5.....	10	25	25	12.....	5	75	85
6.....	10	25	25				
7.....	10	50	30	Average.....	22	47	57

Counts were made of number of lesions, and there were about as many with 10 hours' as with 24 or 50 hours' incubation. Hence, the differences in percentage of leaf area killed were the result of the much larger lesions that developed with water-soaked areas persisting 24 and 50 hours. The great increase in leaf area killed occurred between 10 and 24 hours, and a further increase from 24 to 50 hours had but little effect. The results obtained from this and similar experiments showed that for the development of the destructive epidemic type of wildfire the water-soaked areas must persist for 24 hours or more after inoculation has occurred. Under field conditions, inoculation and infection may not occur for some time after the start of the storm; in fact, many water-soaked areas never become infected, and all traces of water soaking disappear. The persistence factor consequently is very important, and, with both natural epidemics studied, water soaking persisted for about 48 hours.

#### EPIDEMIC WILDFIRE REPRODUCED IN THE FIELD

As a final proof of the relation of water soaking to epidemic wildfire development, this type of disease was reproduced in the field. To accomplish this, plants were sprayed during the early stages of ordinary rainstorms until water-soaked areas appeared on the leaves. Part of the sprayed plants were then inoculated with *Bacterium tabacum*, and part were held as uninoculated checks. Other plants were inoculated but not water-sprayed. Figure 6 shows epidemic wildfire that resulted from the combination of water spraying and inoculation. Figure 7 shows the healthy condition of similar plants that were water-sprayed but not inoculated. Plants inoculated but not water-sprayed developed the usual harmless halo spots.

#### FACTORS MODIFYING SUSCEPTIBILITY OF LEAVES TO WATER SOAKING

##### SURFACE OF LEAF EXPOSED

It has been shown that when leaves are held in a firm position and a strong water spray is applied extensive water soaking can be produced in one-half to 2 minutes. Under field conditions, however, leaves do not become water-soaked so readily. In fact, only the severest storms produce any considerable amount of water soaking, and slight variations in the susceptibility of the leaves result in marked differences in the amount of water soaking and hence in the severity of disease development. Observations during storms indicated that water soaking was greatly facilitated when the lower leaf surface was exposed. The susceptibility to water soaking of upper and lower leaf surfaces was measured under controlled conditions. Half of each leaf was sprayed from above and the other half from below. Table 4 gives the results from such measurements with two sets of leaves—the high-nitrogen leaves, which were very susceptible to disease, and the low-nitrogen leaves, which were very resistant. All the plants from which the leaves were taken were topped low. Even during the severe epidemic of 1933 very little disease developed in the low-nitrogen plots.





FIGURE 6—Epidemic wildfire produced in the field by water soaking leaves with a power sprayer, and inoculating during the progress of an ordinary rain.



FIGURE 7.—Several plants only a few feet distant from those shown in figure 6. These plants were water-soaked with the power sprayer, but they were not inoculated. The water soaking disappeared completely as soon as the weather cleared.

TABLE 4.—*Relative susceptibility to water soaking of upper and lower leaf surfaces on high-nitrogen and low-nitrogen plants*

Leaf no	Time required to water-soak indicated surface of leaf from high-nitrogen plants		Time required to water-soak indicated surface of leaf from low-nitrogen plants		Leaf no.	Time required to water-soak indicated surface of leaf from high-nitrogen plants		Time required to water-soak indicated surface of leaf from low-nitrogen plants	
	Upper	Lower	Upper	Lower		Upper	Lower	Upper	Lower
1.....	Seconds	Seconds	Seconds	Seconds	4.....	Seconds	Seconds	Seconds	Seconds
2.....	90	45	240	180	5.....	105	60	360	360
3.....	70	45	330	270		110	40	300	420
	90	45	235	160	Average..	93	47	293	278

The marked difference in susceptibility to water soaking between leaves from low-nitrogen and those from high-nitrogen plants is to be noted first, and the extreme resistance of the former explains adequately their freedom from epidemic wildfire. The most rapid water soaking was secured with high-nitrogen leaves having the lower leaf surface exposed, the average time being 47 seconds. The same leaves, but with upper surface exposed, required an average of 93 seconds to water-soak.

In another experiment leaves were taken from plants given average topping and fertilization treatment. As in the previous test, half of each leaf was sprayed from above and the other half from below. However, the duration of the spray treatment was 1 minute from below and 2 minutes from above. The data were taken in terms of size of water-soaked areas (table 5).

TABLE 5.—*Relative susceptibility of upper and lower leaf surfaces to water soaking*

Leaf no.	Size of water-soaked area		Leaf no.	Size of water-soaked area	
	Lower surface sprayed 1 minute	Upper surface sprayed 2 minutes		Lower surface sprayed 1 minute	Upper surface sprayed 2 minutes
1.....	Sq. in.	Sq. in.	5.....	Sq. in.	Sq. in.
2.....	16.9	12.26	6.....	30.5	18.8
3.....	24.2	15.0		25.2	9.7
4.....	12.5	4.5	Average.....	20.0	11.3
	10.5	7.5			

A comparison of average results shows that a 2-minute treatment of upper surfaces produced 11.3 square inches of water soaking, while a treatment half as long of lower surfaces gave 20 square inches. Similar experiments have been conducted with many lots of leaves. Lower-surface exposures of equal duration have yielded water-soaked areas as much as five times as large as those obtained with upper-surface exposures. Hence it may be concluded that if leaves are so turned that the under surface is exposed to the rain, water soaking is greatly facilitated.

Earlier in this study it was shown that the normal leaf, not water-soaked, is more readily infected by applying the inoculum to the

lower leaf surface, and that the upper surface of mature leaves is especially difficult to infect. In view of this, it seemed advisable to consider the effects of different combinations of water-spray treatments of upper and lower leaf surfaces with inoculations, as shown in table 6. This experiment was repeated several times with similar results.

TABLE 6.—*Effect of different combinations of water-spray and inoculation treatments on disease development in old and in young leaves*

Leaf surface treated		Old leaves		Young leaves	
Water-sprayed	Inoculated	Average number of lesions	Average percentage of leaf area killed	Average number of lesions	Average percentage of leaf area killed
None.....	Upper.....	1	0	24	1.5
Upper.....	do.....	31	47	83	50
Lower.....	do.....	65	80	200+	80
None.....	Lower.....	6	1	85	3.5
Lower.....	do.....	118	100	200+	90
Upper.....	do.....	69	87	200+	80

In comparing results obtained with old and with young leaves, it is to be noted that, although all leaves were equally water-soaked in the beginning and saturated air conditions were maintained during incubation, the water-soaked areas were always retained more completely by the more mature leaves. This difference is associated with slightly higher values for percentage of leaf area killed in the case of the mature leaves. The chief point brought out by table 6, however, is that there is little relation between susceptibility to infection of the normal leaf and the development of epidemic wildfire. The old leaves that were infected with difficulty when not water-soaked were destroyed by the disease after water soaking even more completely than were the young leaves that were readily infected. Table 6 shows also that, regardless of which leaf surface was water-sprayed and which was inoculated, all developed the epidemic type of disease; the minimum of leaf area destroyed was 47 percent and the maximum 100 percent. Without water soaking, the same inoculations produced only the halo type of wildfire, and the minimum and maximum figures for area killed were 0 and 3.5 percent, respectively.

#### LEAF INJURIES

In the field, after storms, water-soaked areas were frequently observed surrounding old wildfire lesions. Similar water-soaked areas were noted about fresh mechanical injuries, but old injuries or insect punctures had no effect. Experiments under controlled conditions confirmed these observations and showed conclusively that both fresh mechanical injuries and wildfire lesions of any age materially reduce leaf resistance to water soaking. Toward the latter part of the summer medium- to small-sized wildfire lesions often are very numerous, and their function in supplying bacteria for further disease development is obvious. The data in table 7 show their importance in increasing susceptibility of leaves to water soaking. In this experiment pairs of adjoining healthy and diseased leaves from the same plant were compared.

TABLE 7.—*Effect of old wildfire lesions on the susceptibility of leaves to water soaking*

Leaf pair no.	Size of water-soaked area of leaf with—				Leaf pair no.	Size of water-soaked area of leaf with—			
	Upper surface sprayed 1½ minutes		Lower surface sprayed ¾ minute			Upper surface sprayed 1½ minutes		Lower surface sprayed ¾ minute	
	Diseased leaf	Healthy leaf	Diseased leaf	Healthy leaf		Diseased leaf	Healthy leaf	Diseased leaf	Healthy leaf
	<i>Sq. in.</i>	<i>Sq. in.</i>	<i>Sq. in.</i>	<i>Sq. in.</i>		<i>Sq. in.</i>	<i>Sq. in.</i>	<i>Sq. in.</i>	<i>Sq. in.</i>
1-----	16.5	3.2	7.75	7.75	7-----	5.5	3.2	6.5	2.0
2-----	8.0	4.0	7.3	6.7	8-----	12.0	4.5	8.3	9.0
3-----	5.5	6.75	5.75	1.0	9-----	19.0	12.5	17.9	18.6
4-----	6.25	6.25	4.0	4.2	10-----	12.0	2.6	11.0	12.0
5-----	15.0	7.5	7.7	3.4					
6-----	11.0	5.5	12.5	12.5	Average.	11.1	5.0	8.9	7.7

The 10 pairs of leaves used in this experiment were selected with special care to insure that they were as nearly alike as possible, except for the presence or absence of wildfire lesions. These lesions, however, were neither numerous nor large. The outstanding development was the marked increase in susceptibility of upper leaf surfaces to water soaking, as a result of the presence of disease lesions. This correlates with the field observation that diseased leaves often show extensive water soaking while adjoining healthy leaves are but little affected.

Indirectly these experiments indicated that the intercellular water is driven in from the outside rather than exuded from the cells, and that many wildfire lesions which appear inactive are probably enlarging slowly, since otherwise they should behave in the same manner as old mechanical injuries.

#### LEAF MATURITY

When tobacco plants are either topped high or not topped at all, the basal leaves may be mature while the tip leaves are yet very young. These differences in leaf maturity have been associated with marked differences in the susceptibility of the leaves to water soaking and to epidemic wildfire, and, while nutrition affected the total amount of disease, it did not alter the base-tip relation. Thus, field counts following the 1933 epidemic showed in the low-nitrogen plots averages per plant of one basal leaf killed, the next two or three leaves severely injured, and from there to the tip a rapid decrease in injury to a bare trace. The upper three-fourths of these plants showed abundant infection, but the lesions were almost entirely of the harmless halo type. Adjacent plots that received no potash averaged four basal leaves killed and the next four or five severely injured, only the tip quarter of the plant escaping injury. These differences in wildfire injury on different portions of the same plant were clearly associated with differences in water soaking. The areas were much larger on basal leaves and persisted much longer.

Susceptibility to water soaking of different leaves from the same plant was measured in the usual manner. Table 8 shows the results from one experiment in which a half-minute treatment of the lower leaf surface was used.

TABLE 8.—*Relative susceptibility to water soaking of basal, middle, and tip leaves*

Plant no	Area water-soaked			Persistence of water-soaked condition		
	Basal leaves	Middle leaves	Tip leaves	Basal leaves	Middle leaves	Tip leaves
	<i>Sq. in.</i>	<i>Sq. in.</i>	<i>Sq. in.</i>	<i>Hours</i>	<i>Hours</i>	<i>Hours</i>
1.....	27.5	10.8	0.0	5.5	0.25	0.0
2.....	18.3	1.0	.2	2.75	2.25	1.0
3.....	12.8	1.0	.3	5.0	2.5	.5
4.....	21.3	2.3	.2	4.5	2.0	1.0
5.....	13.0	1.3	.4	4.0	1.0	.75
6.....	11.5	1.5	.2	4.75	1.25	.75
Average.....	17.4	2.98	.22	4.42	1.54	.67

The results given in table 8 show clearly that from the base to the tip of the plant the size of the water-soaked areas decreases rapidly, as does also the length of time that the water-soaked condition persists. These differences are quite adequate to explain the observed difference in disease development. Additional studies of the relation of leaf maturity were conducted by inoculating plants of different ages in the greenhouse and by making successive plantings in the field. In such tests the older plants were severely injured by the epidemic type of wildfire, while the young plants, though freely infected, suffered but little injury.

The data given in table 8 were secured by water spraying lower leaf surfaces. Treatments applied to the upper leaf surfaces yielded results quite as marked. Thus in one experiment, in which a 45-second treatment was used, mature leaves developed large water-soaked areas that persisted for an average period of 5 hours and 50 minutes. The much smaller areas on the immature leaves persisted for an average period of 37 minutes. In conclusion, it is to be noted that while young leaves tend to escape water soaking, and hence disease damage, yet under very severe storm conditions both young and old leaves may become heavily water-soaked. When this happens, there is no difference in the degree of subsequent disease injury.

#### HEIGHT OF TOPPING

The height at which plants are topped has a consistent and marked effect on their susceptibility to leaf spot. It is probable that the primary effect of topping is on leaf maturity. Thus, as has been pointed out in the discussion of leaf maturity, plants that either are not topped or are topped high bear old leaves at the base and young leaves at the tip. Topping hastens the maturity of the tip leaves that remain, and the lower the plants are topped the greater is the tendency for the remaining leaves to mature together. The effect of such low topping on disease susceptibility is illustrated by a greenhouse experiment in which all leaves were uniformly water-sprayed and inoculated; the results are summarized in table 9.

TABLE 9.—*Effect of topping on leaf spot injury*

Location of leaves on plants	Leaf area killed		
	Low-topped	High-topped	Not topped
	<i>Percentage</i>	<i>Percentage</i>	<i>Percentage</i>
Base.....	79.3	67.9	76.0
Middle.....	81.7	36.6	43.4
Tip.....	75.4	9.4	7.1

The low-topped plants averaged 6 leaves, the high-topped 13 leaves, and those not topped 16 leaves. Infection was abundant on all leaves, and the differences in percentage of leaf area killed were due to differences in size of lesions. It is noteworthy that all the leaves of the low-topped plants responded alike to the disease and were highly susceptible, while in the other plants there were marked differences in susceptibility between the basal and the tip leaves.

The effect of topping on disease was equally marked in the greenhouse and in the field. The data collected from the field plots at Upper Marlboro, Md., following the epidemic of 1933, were of special interest.

TABLE 10.—*Influence of topping on wildfire development in the field in 1933*

Location of leaves on the plants	Plants topped low			Plants not topped		
	Average number of lesions	Diameter of lesions <sup>1</sup>	Area killed	Average number of lesions	Diameter of lesions	Area killed
		<i>Inches</i>	<i>Percent</i>		<i>Inches</i>	<i>Percent</i>
Base.....	800+	0.44	75	800	0.13	10
Middle.....	650	.28	40	650	.09	5
Tip.....	600	.22	25	375	.06	1

<sup>1</sup> The lesions measured had space enough for full development; many lesions were so close together that they soon coalesced.

The low-topped plants averaged 13 leaves; the plants that were not topped, 25 leaves. The figures for the average number of lesions per leaf are given because they show clearly that all leaves were freely infected, and the differences in disease loss were the result of the much larger size of the lesions on the leaves of the plants topped low. It is to be noted that in this field experiment even the basal leaves of the plants that were not topped suffered but little injury.

Field observations indicated that the differences in disease susceptibility induced by topping were the result of differences in susceptibility to water soaking, which either facilitated or inhibited the development of the epidemic type of wildfire. Figure 8 illustrates this effect of topping on type of disease developed. To measure susceptibility to water soaking under actual field conditions, selected plants were treated with a power spray during the course of an ordinary rain, and counts of number of water-soaked areas persisting were made when the weather began to clear, 19 hours later. The average number of areas on low-topped plants was 15.3 per leaf; on high-topped plants, 0.2 per leaf. In another test of susceptibility to water soaking, 21 leaves were picked from low-topped plants and an equal number

from plants that were not topped. All were subjected to a uniform water-spray treatment, and data were taken on both size and persistence of water-soaked areas. The comparative figures for water-soaked areas were as follows. For size: Low-topped, 15.45 square inches; high-topped, 7.15 square inches. For persistence: Low-topped, 5.8 hours; high-topped, 3.9 hours. The water spray in this experiment was applied to the lower leaf surface. Previous experiments had indicated that where differences in susceptibility to water soaking were involved these differences tended to be greater with

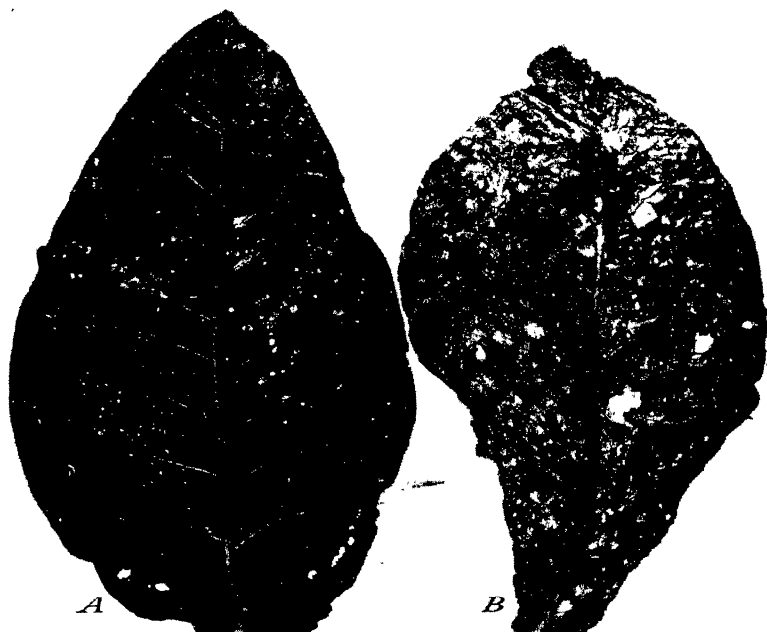


FIGURE 8.—A, Halo wildfire on a plant that was not topped. B, Epidemic wildfire on an adjacent low-topped plant. Leaves on plants that are not topped water-soak with difficulty and, except under the most severe storm conditions, are but slightly injured. Leaves on low-topped plants water-soak easily and the areas persist, hence their susceptibility to the epidemic type of disease.

upper leaf surfaces. The results of an experiment summarized in table 11 tended to support this view.

TABLE 11.—*Influence of topping on the susceptibility of leaves to water soaking*

Leaf no.	Water-soaked areas				Leaf no	Water-soaked areas			
	Lower surface sprayed 1 min- ute		Upper surface sprayed 2 min- utes			Lower surface sprayed 1 min- ute		Upper surface sprayed 2 min- utes	
	Low- topped	Not topped	Low- topped	Not topped		Low- topped	Not topped	Low- topped	Not topped
	<i>Sq. in.</i>	<i>Sq. in.</i>	<i>Sq. in.</i>	<i>Sq. in.</i>		<i>Sq. in.</i>	<i>Sq. in.</i>	<i>Sq. in.</i>	<i>Sq. in.</i>
1-----	14.3	12.0	10.9	7.3	6-----	24.0	8.3	6.3	2.5
2-----	22.3	10.0	11.8	2.5	7-----	19.3	11.1	6.0	1.3
3-----	7.7	4.0	2.8	1.3	8-----	10.0	6.7	9.5	.0
4-----	5.3	1.0	4.3	1.0					
5-----	30.0	12.3	15.3	7.5	Average	16.6	8.2	8.4	2.9



The data presented in table 11 show that when the water spray was applied to the lower leaf surface the areas produced were twice as large on leaves from low-topped plants; when the upper surfaces were treated, the areas on leaves from low-topped plants were three times as large. The data show also the wide variation in size of water-soaked areas produced on supposedly similar leaves. Thus on leaves from the low-topped plants the range was from 5.3 to 30.0 square inches. Wide variations such as this have been the rule in the field, where they were first assumed to be due entirely to unequal exposure. Evidently leaves that are close together and look alike may yet possess differences sufficient to have a marked effect on the amount of water soaking. This explains why apparently similar leaves may be either slightly or severely injured by the disease.

Before concluding the discussion of topping, attention should be called to several modifying factors that are operative under field conditions and that tend to increase the differences previously noted. In the first place, leaves on low-topped plants are stiff and brittle while leaves on plants not topped are thinner and more pliable. Consequently, leaves on low-topped plants offer a much firmer resistance to wind and rain, and, when they do bend, mechanical injuries are frequent. In the controlled experiments all leaves were held against a padded board and much care was taken not to injure them. This procedure eliminated the field conditions mentioned. A second field factor, facilitating the water soaking of leaves on low-topped plants, was the tendency of these leaves to turn over in the normal course of growth, and so to expose the under surface. The counts from field plots given in table 12 indicate the comparative number of upturned leaves found under different fertilization and topping treatments.

TABLE 12.—*Topping in relation to number of upturned leaves<sup>1</sup> on plants grown with various quantities of nitrogen*

Fertilizer treatment	Upturned leaves on—	
	Low-topped plants	Plants not topped
	Number	Number
No nitrogen.....	3	0
Regular nitrogen..	12	7
High nitrogen.....	56	26

<sup>1</sup> Total for 40 plants in each case.

The counts of leaves turned up were the totals in each case for 40 plants. Since there were twice as many leaves on the plants that were not topped, the actual differences, in proportion to total number of leaves, were much greater even than indicated in table 12. The greater susceptibility of upturned leaves to water soaking results in these being the first to develop epidemic wildfire, and it is interesting to note that low topping and high-nitrogen fertilization, the combination that produces maximum leaf susceptibility, also produces the maximum of upturned leaves. This factor, then, has an appreciable effect in making a susceptible planting even more susceptible.

## FERTILIZATION

In the nutrition experiments at Upper Marlboro, the development of wildfire has been consistently modified by variations in fertilization. The plots most susceptible to disease were those that were topped low and received a high-nitrogen fertilizer, those receiving no potash, and those receiving no fertilizer at all. Resistance was clearly correlated with low-nitrogen and high-potash fertilization. Figure 9 shows the marked effect of fertilization on disease development. Just as in the case of other factors, these differences have been associated with modifications in leaf-water relations, by both field observation and controlled experimentation. Table 13 clearly shows the marked effects of fertilization on disease development.

TABLE 13.—Wildfire development as modified by fertilization, Upper Marlboro, Md., 1933

Fertilizer treatment	Average number of lesions per leaf	Average number of leaves per plant				Estimated total damage
		Killed	Severely injured	Slightly injured	Not injured	
No fertilizer.....	( <sup>1</sup> )	3	7	3	0	Percent 80
No potassium.....	850	2	5	6	0	55
No phosphorus.....	350	0	2	8	4	20
No nitrogen.....	150	0	0	4	10	3
Complete fertilizer.....	500	0	3	10	4	25

<sup>1</sup> Lesions coalesced.

Table 14 shows the susceptibility to water soaking of leaves from the above-mentioned plots. The values in each case are averages for five leaves, and the experiment was duplicated with similar results.

TABLE 14.—Susceptibility of the leaves to water soaking as affected by fertilization

Fertilizer treatment	Average <sup>1</sup> size of water-soaked areas	Average <sup>1</sup> persistence of water-soaked areas	Fertilizer treatment	Average <sup>1</sup> size of water-soaked areas	Average <sup>1</sup> persistence of water-soaked areas
	Sq. in	Hours		Sq. in.	Hours
No fertilizer.....	21.3	1.9	No nitrogen.....	3.9	0.2
No potassium.....	22.8	2.75	Complete fertilizer.....	12.58	1.3
No phosphorus.....	11.5	1.2			

<sup>1</sup> Average for 5 leaves.

The correspondence between susceptibility to water soaking, as indicated either by the size or persistence of water-soaked areas (table 14), and disease susceptibility, as indicated by the percentage of estimated total damage (table 13), is so apparent as to require no comment. The only variation from the expected was that, with no fertilizer, water-soaking values were not so high as would be anticipated. Additional evidence that fertilization modifies disease development through its effect on water relations may be seen in table 4, where susceptibility to water soaking of leaves from high- and low-nitrogen plants is compared. The former were disease-susceptible and easily water-soaked, the latter disease-resistant and difficult to

water-soak. The effect of high-potash fertilization in retarding disease development and of low potash in promoting disease development has been mentioned, and this, too, is correlated with water



FIGURE 9.—Representative half leaf (A) from a no-nitrogen plot and (B) from a no-potash plot. The former is resistant to water soaking, the latter susceptible. The epidemic wildfire shown in B followed the severe August storm of 1933.

relations. Thus it was found that in the absence of potash the average size of the water-soaked areas was 28.4 square inches and their average persistence 3.2 hours, whereas when potash at the rate of

240 pounds per acre was used the average size of the water-soaked areas was only 5.8 square inches and their average persistence 0.1 hour.

In conclusion, however, it is to be noted that these marked effects from fertilization were obtained at Upper Marlboro, with a very light sandy soil, and that the treatments indicated had been repeated for a number of years. Experiments elsewhere, with more fertile soils and with the different treatments applied to but a single year's crop, failed to show marked differences in plant growth, in disease development, or in susceptibility to water soaking, except in the case of nitrogen. Hence, while it may be safely concluded that excessive nitrogen applications will generally produce increased susceptibility to wildfire, both the nature of the soil and previous fertilization evidently will determine the effectiveness of potash. For example, in Pennsylvania the soil type is such that plants absorb but little potash, regardless of the amount applied.

#### DISCUSSION

This investigation, which was undertaken to elucidate the etiology of the destructive type of leaf spot that attacks tobacco in the Maryland-Pennsylvania area, resolved itself into a study of the relation of storms to the development of epidemic wildfire. The relation of storms to the development of destructive wildfire epidemics had been reported many times and had been explained on the basis of dissemination. Thus Chapman and Anderson (4, p. 71) early showed that the organism could be readily spread by rain spattering, and their explanation of storm effect was as follows:

It has been noted by all investigators of the disease and by tobacco growers that rapid spread invariably follows heavy rains. When the raindrops fall on the diseased spots, the bacteria float out into the water and successive drops splash them to other leaves of the same plant or neighboring plants. If the rain is accompanied by wind, the drops are carried farther and the spread is greater to the windward of diseased plants. \* \* \* These two agents (wind and rain) are undoubtedly the most potent of all the factors involved in dissemination.

While this conception was generally accepted, certain other workers offered different hypotheses. Thus Johnson and Fracker (11, p. 13) say:

Storms, especially beating rains, however, have a very important relation to wildfire in that they favor infection to a high degree. The bacteria are often unable to infect leaves except through slightly wounded tissue, such as may be produced by beating rain, although with rapidly growing tobacco, moisture in itself often suffices for considerable infection. The facts are, generally, that heavy infection and damage practically depend on storms or continued rains.

Still another interpretation of storm effect was given by Clinton and McCormick (7, pp. 387, 388) as follows:

The greatest injury comes just after the disease spreads over the plants during the period of wet weather and the sun suddenly shines again. The halo spots then turn to brown irregular burn-like areas. This transformation often takes place quickly and so probably is largely a mechanical injury to the badly infected tissues.

The statements given in the last two quotations appeared in 1922 and have not been referred to since then. More recently Böning (2, pp. 12-18) has advanced the hypothesis that rains affect disease development by modifying plant nutrition. He suggested that during dry weather nitrates accumulate in the surface soil, and that the rains

then wash the nitrates down to where the roots can absorb them. In this manner the nitrogen intake is increased and the leaves are made more susceptible.

The negative results reported in the first part of this paper show quite conclusively that the development of epidemic wildfire cannot be adequately explained by dissemination, leaf injury, or nutrition. Inoculations, in which these and all other known environmental and plant factors were widely varied, always resulted in the formation of small dead areas, rarely more than one-fourth inch in diameter, which were surrounded by yellow halos. Even large numbers of these halo lesions caused but little actual leaf damage.

The first definite clue to the factor responsible for the development of the destructive epidemic type of wildfire was the discovery that during very severe rain and wind storms numerous water-soaked areas appeared scattered over the leaves. These were external evidence that the intercellular spaces below were flooded. So far as the writer is aware, the only previous observation of these water-soaked spots was made by Valteau and Johnson (15), who associated them with what they considered to be nonparasitic blackfire. The writer found that these water-soaked areas facilitated invasion by the bacteria but that by far their most important effect was in facilitating the spread of the bacteria through the leaf tissues after infection had occurred. This penetration was so rapid that large areas of diseased leaf began to wilt and die after 48 hours. The death of the diseased tissues was most rapid after the sun appeared, just as suggested by Clinton and McCormick (7), but only because the sun hastened the necrosis. Following this initial rapid-development phase, the bacteria were able to invade and kill new tissues for a period of 10 to 14 days, and this resulted in a secondary and more gradual phase of lesion enlargement.

It is of interest now to consider why the lesions of epidemic wildfire differ so materially in appearance from those of halo wildfire. The conspicuous feature of the latter is the yellow halo surrounding the small central dead area, and the confusion regarding the cause of epidemic wildfire was due (1) to the rapid development of the lesions, (2) to their large size, and (3) to the fact that they showed little or no halo effect around the margins. The halo is caused by the toxin excreted by the bacteria diffusing out ahead of the organism into adjacent living cells and destroying the chloroplasts, as has previously been described (6). Hence halo lesions result when the bacteria remain alive for some time in an area but are unable to rapidly invade and destroy the adjacent cells, thus giving the toxin time to diffuse out ahead. With the aid of water soaking, however, tissue invasion proceeds at such a rate that the adjoining tissues are killed before there is time for the development of halos. Thus the appearance of halo lesions, instead of being proof of parasitic activity, is really proof that bacterial invasion has been checked successfully by the host. In this connection it is to be recalled that the *Bacterium tabacum* toxin is able to produce halo spots on inoculation into the leaves of many plants, but the bacteria are able to parasitize only tobacco. It now appears that they are able to successfully parasitize tobacco only under very special conditions, i. e., when the resistance of the leaf has been broken down by water soaking. Hence we have the unusual situation of a host that is highly resistant to the parasite under all

ordinary conditions but which can be rendered highly susceptible through the action of beating rain. In addition to this major storm effect on host susceptibility, it is not questioned that spattering water aids greatly in the dissemination of the organism, and that this dissemination under favorable conditions for infection is a necessary factor in the development of the leaf spot epidemic. Growers have been inclined to attribute leaf spot damage directly to storms, but this is proved to be incorrect by the fact that, in the absence of the organism, leaf spot does not develop, regardless of storm severity.

It has also been shown that such practices as high-nitrogen fertilization and low topping do not in themselves make plants susceptible to epidemic leaf spot but that by making the leaves more susceptible to water soaking they act indirectly. High humidity, also, does not produce epidemic wildfire, but high humidity following water soaking helps to maintain the water-soaked areas, and epidemic leaf spot development requires that these areas persist at least 24 hours. Various other factors that affect disease development in the field have also been examined and have been found to exercise these effects by modifying water relations in the leaves. Thus, during epidemics of wildfire the leaves on the windward side of plants were most severely injured, and this was supposed to be due to the greater exposure of these leaves to infection. What actually happens is that the wind bends the plants and turns up the leaves on the windward side so that under surfaces are exposed. These leaves then become heavily water-soaked, while leaves on the leeward side, with upper surfaces exposed, do not water-soak so readily.

The mechanics of water soaking has not been investigated, but certain suggestive evidence may be mentioned. In the first place, leaves do not readily water-soak when dipped in water or when exposed to a gentle spray, but a hard spray is very effective, indicating that it is not merely water, but forcibly applied water that is required. It is possible that beating rain might alter the permeability of interior cells and cause them to excrete liquid into the spaces. However, there are indications that the water may come from outside. For example, disease lesions and fresh breaks in the leaf surface are soon surrounded by water-soaked tissues, suggesting that the water was driven in through the openings thus afforded. The structure of the leaf indicates that the lower leaf surface would be more easily penetrated than the upper. The question of how low topping and high-nitrogen fertilization make leaves more susceptible to water soaking has not been studied, other than to note that these treatments modify leaf structure materially. These problems can be definitely solved only by further investigation.

It has been demonstrated that water soaking favors rapid spread of the bacteria through the leaf tissues, and this raises the question as to how this spread takes place. Hill (10) has reported that *Bacterium tabacum* moves in the form of a zoogloea, which would imply a rather slow penetration. It is probable, however, that he worked with the usual halo type of lesion. The writer has prepared sections of infected water-soaked tissues, and found the bacteria widely scattered through the intercellular spaces, with no indication of zoogloea. Hence it seems likely that under epidemic conditions the bacteria spread along the liquid pathways as free-swimming cells,

and that this is the reason for the resulting rapid and extensive disease development.

In view of the dependence of epidemic wildfire on storms, the question naturally arises as to whether some other plant diseases may not be similarly influenced. A search of the literature has failed to disclose any evidence that the storm water-soaking relation has been observed by others, but several investigators have noted the role of intercellular liquid in facilitating infection and disease development. Thus Riker (13) found that, when he inoculated stems with *Bacterium tumefaciens* by wounding, the tissues around the wounds became water-soaked. The resulting galls never extended beyond the water-soaked areas. Similarly, Meier (12) reports that infection with *Bact. campestris* was restricted to the margins of the leaves because only there did the hydathodes provide a liquid pathway for the bacteria. The writer, however, has previously reported (5) that infection with *Bact. campestris* occurs at any point on the leaf under some conditions, and that such epidemic outbreaks of black rot were preceded by the appearance of numerous water-soaked areas scattered over the leaf. The water-soaked areas in this case were formed during warm humid nights, without the aid of storms. The subsequent development of black rot lesions followed the course here described for epidemic wildfire.

It seems likely that further investigation will show that the development of water-soaked tissues as the result of storms or other causes is an important factor in the epidemiology of a number of diseases. Evidence not yet published shows that this is true of another tobacco leaf spot disease, blackfire, caused by *Bacterium angulatum*.

Several hypotheses advanced by other workers to explain resistance and susceptibility to wildfire are quite different from the conception herein presented, which is, briefly, that the normal tobacco leaf is highly resistant to invasion but that by water soaking it is rendered highly susceptible, and that various cultural and nutritional factors do not make cells more or less easy to attack but do make tissues more or less easy to water-soak. Dufrenoy (8) attributes to the host tissues ability to set up a definite defense mechanism and suggests that the lesions cease to spread because of phenolic compounds that are formed in the cells surrounding the lesions. However, the writer finds that under field conditions the tissues adjoining old lesions are very likely to be first invaded when a subsequent epidemic develops. This is correlated with the fact that the tissues adjoining old lesions are easily water-soaked; there is no indication that they are fundamentally more or less susceptible. Böning (3) associates wildfire susceptibility and resistance with nutritional balance in the leaves but offers no satisfactory explanation of how so-called unbalanced leaf nutrition would be effective in inducing resistance and susceptibility.

The results obtained by the writer do not indicate that tobacco-leaf tissues differ to an important degree in their inherent susceptibility or resistance to attack by *Bacterium tabacum*, since if intercellular liquid is present all are about equally and completely susceptible and if it is not present all are resistant. It seems, consequently, that apparent leaf resistance is merely a matter of disease escape, with water relations as the immediate deciding factor. Also, the assumption generally made that susceptibility to infection indi-

cates susceptibility to the epidemic type of disease does not hold. Young leaves were susceptible to infection but resistant to epidemic wildfire in the field because they were not readily water-soaked.

In conclusion, it should be noted that, theoretically, effective wildfire control can be accomplished (1) by eliminating the organism, (2) by protecting the plants from storms (as is actually done on a small scale where crops are grown under cloth), or (3) by securing resistance either directly or through resistance to water soaking. At present, with the organism generally distributed in most fields throughout the Maryland-Pennsylvania area, it may be safely predicted that destructive wildfire epidemics will follow severe late summer wind and rain storms, and when such epidemics occur the damage to the plants will be modified by the various factors that increase or diminish leaf resistance to water soaking.

It is believed that the experiments herein reported adequately explain how *Bacterium tabacum* is able to cause the usual and relatively harmless halo lesions and also the destructive epidemic type of the disease and show that there is no evidence of the existence of a similar nonparasitic leaf spot.

#### SUMMARY

Tobacco leaves are readily infected by *Bacterium tabacum*, but under ordinary conditions invasion is limited to small areas. The usual result of infection is a dead spot, one-fourth of an inch or less in diameter, surrounded by a yellow halo. These halo lesions do little damage, but have been regarded heretofore as typical wildfire.

The destructive epidemic type of wildfire is characterized by large lesions that develop very quickly and show little or no halo effect. The question has been raised as to whether this type of disease might be wholly or partly nonparasitic in nature.

Inoculation experiments designed to reproduce the epidemic type of lesion by varying the nutrition of the plant through fertilization and topping resulted only in the halo type of lesion. In other experiments consideration was given to age of leaves, methods of inoculation, and incubation conditions, particularly temperature and humidity, but the lesions obtained were all of the halo type.

Observations during storms showed that the tobacco leaves develop water-soaked areas owing to the flooding of intercellular spaces. When these areas become infected the bacteria multiply and spread through the tissues with extraordinary rapidity, and the epidemic type of lesion results.

Without water soaking, about a week was required for the development of the small halo lesions; but with water soaking, large epidemic-type lesions were produced in 48 hours. In controlled inoculations, water soaking increased the size of lesions by more than 6,000 percent.

Large water-soaked areas tended to produce large disease lesions.

Persistence of water-soaked areas for 24 hours or more was essential for epidemic disease development.

With the aid of a power sprayer to water-soak the leaves, it was possible to reproduce the typical epidemic wildfire under field conditions. Susceptibility of leaves to water soaking, however, was found to be modified by many factors, and these differences account for wide differences in disease damage.



Exposure of the lower leaf surface greatly facilitated water soaking, as did also the presence of fresh mechanical injuries or wildfire lesions of any age.

Mature basal leaves became water-soaked more readily than did young tip leaves on the same plant.

Low topping increased susceptibility of the leaves to water soaking, while topping high or not topping at all had the opposite effect.

High-nitrogen and low-potash fertilization both increased susceptibility of the leaves to water soaking.

There was little relation between susceptibility to infection of the normal leaf and the development of epidemic wildfire. Mature leaves were infected with difficulty when not water-soaked, but after water soaking they were more severely damaged than young leaves that were readily infected.

Since all types of leaves were highly resistant to bacterial invasion when not water-soaked and highly susceptible when water-soaked, it is concluded that susceptibility and resistance to *Bacterium tabacum* is primarily a question of water relations, and that the halo type of wildfire is the response to infection of the normal leaf and the epidemic type of wildfire is the response of the leaf after its resistance has been broken down by water soaking.

Epidemic wildfire in the Maryland-Pennsylvania area, consequently, is caused by *Bact. tabacum*, and its occurrence is conditional on storms of sufficient severity and duration to produce and maintain water-soaked areas on the leaves. The amount of water soaking, and hence the amount of disease damage, is modified by prevailing cultural and fertilization practices; low topping in combination with high-nitrogen fertilization, which is the practice in Pennsylvania, favors maximum disease development.

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# BEHAVIOR OF THE ORDINARY TOBACCO MOSAIC VIRUS IN THE SOIL<sup>1</sup>

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## INTRODUCTION

It is fairly evident that control of the ordinary tobacco mosaic disease (*tobacco virus 1*) (4)<sup>2</sup> may be accomplished with considerable success through the application of all of a number of precautionary measures that have been recommended for the purpose. These measures are directed chiefly against introduction of the virus into the field from various sources where it may be harbored for long periods, and also against further dissemination of the disease once it has gained entrance into the crop. The means by which the virus, once introduced, is spread in the field are comparatively well understood and may be prevented in considerable degree. The relative importance of the various sources of initial infection is, however, not so clear. The multiplicity of ways in which the virus may persist for prolonged periods apart from the living tobacco plant renders the problem a complex one, requiring intensive study in its various phases. One phase of the problem in particular need of greater consideration than has been accorded to it relates to the harboring of the virus in the field soil.

The present paper deals with the results of laboratory and greenhouse studies on the persistence and inactivation of the ordinary tobacco mosaic virus in the soil under various conditions. These investigations have shown that the virus may survive in soils for periods of 1 year or more, but that it is subject to relatively rapid inactivation under certain natural conditions. This inactivation is not sufficient, however, to eliminate soil infestation as a factor in tobacco mosaic infection, the more practical aspects of which it is proposed to discuss in a later contribution from this laboratory.

## EARLIER INVESTIGATIONS

A brief survey of the early literature dealing with overwintering of the tobacco mosaic virus is presented in a previous paper from this laboratory (6). Attention is called to the fact that, whereas the earlier observers of tobacco mosaic believed the soil to be a significant source of infection, the later trend of opinion was away from this conception and in favor of overwintering of the virus in perennial hosts, with subsequent dissemination by aphids. The latter hypothesis has now been practically eliminated, however, so far as ordinary tobacco mosaic is concerned (2). More recently, considerable emphasis has

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<sup>2</sup> Reference is made by number (italics) to Literature Cited, p. 294.

been placed on infection occurring during cultural operations through the use of contaminated tobacco by workers (7, 9).

In 1929 Johnson and Ogden (6) presented data showing that the tobacco mosaic virus overwintered naturally in the soil under Wisconsin conditions, and that tobacco transplanted to heavily infested soils developed much higher percentages of infection than did similar plants set in virus-free or slightly infested soils. While other sources of natural infection were recognized, such as tobacco or tobacco refuse, transferred by man, animals, or wind to seedbed or field, soil infestation was regarded as being of major significance.

As a result of various tests performed in North Carolina, Lehman (7) also considered soil infestation to be responsible for some direct infection of the tobacco crop, though in his trials the proportion of plants becoming diseased was too low to account for the high percentages of tobacco mosaic infection common to that section.

The wide range of conditions under which tobacco is grown may be expected to influence the origin and amount of mosaic infection in different districts. However, it appears clear that the data now available are not sufficient to permit definite conclusions to be drawn as to the relative importance of the various sources of infection in the epidemiology of the disease.

#### MATERIALS AND METHODS

The soils in which the behavior of the tobacco mosaic virus has been studied were chiefly "tobacco" soils. Some of these were Wisconsin soils, obtained in relatively large amounts from various parts of the State. In addition, representative smaller lots, up to three in number, were obtained from each of the tobacco-growing States through the courtesy of local, State, or Federal workers, to whom thanks are due. Several other types of soil were also used, including Superior red clay, peat, greenhouse compost, some very acid soils, and sand, so that an almost complete range was available with respect to physical properties, chemical fertility, and reaction.

All soils used are listed in table 3 under the name of the State from which they were obtained. Where lots were obtained from more than one locality in a State, they are distinguished by addition of a letter indicating the particular locality or other distinctive feature. For example, Tennessee (S) soil came from Springfield, Tenn., and Wisconsin (E) soil from Edgerton, Wis. Where more than one lot of soil was obtained from the same locality, designation is made by number, i. e., South Carolina (1), (2), and (3). The soils are listed in the table in order of their total colloidal content. The type to which each soil belongs is also indicated, together with certain other pertinent information.

The material used as soil inoculum was collected mainly from tobacco plants infected with *tobacco virus 1* in the field in September 1934. About 12 liters of juice were extracted from diseased leaves and suckers and stored in a refrigerator at 5° C. until needed for use. In addition, considerable quantities of diseased roots, stubble, stalks, sucker stems, and leaves were cut up into small portions of the required size, the leaves in very small pieces, the other parts in sections about 1½ inches long. These were thoroughly air-dried and stored in a dry cupboard at room temperatures to serve as stock material fairly uniform in virus concentration.

White enamel refrigerator pans of about 5-kg capacity, with covers, were selected as containers for most of the experiments. These pans were chosen, apart from their convenience in handling, chiefly because they allowed approximate reproduction of the conditions of soil aeration and moisture existing at soil depths of about 4 to 8 inches. Soil stored in the covered pans lost moisture only very slowly. The usual procedure was to place 2 kg of air-dried soil in each pan and to bring the soil to approximately one-third of the water-holding capacity by adding 200 cc of virus extract diluted to 1 to 1 with water, together with as much additional water as was required. Thus, the effective dilution of the virus in the soil lots was regarded as being about 1 to 20. After standing for sufficient time to allow the moisture to be absorbed by the soil, the contents of the pan were turned out and thoroughly mixed by hand, in order to insure more even distribution of the virus. The pan and contents were then weighed and later brought up to the original weight at regular intervals by the addition of water.

The dry plant tissues used for soil inoculum were merely added to the moist soils in the proportion of about 1 part to 25 parts of soil, and allowed to remain there until sampled. The infested soils were placed in storage according to requirements as to environment and tested every week, fortnight, or month for virus concentration. Various modifications of this general plan of experimentation were introduced according to circumstances, and these will be briefly discussed in connection with individual experiments.

The presence and relative concentrations of tobacco mosaic virus in any lot of soil was determined by means of the local-lesion method, inoculations being made into *Nicotiana glutinosa* or into the hybrid, *N. glutinosa*  $\times$  *N. tabacum* (5). For the type of investigation planned, this method offers many advantages over the older method of inoculation to *N. tabacum*. Use of the hybrid in place of *N. glutinosa* was also found to increase the range and reliability of the determinations and was adopted for most of the experiments.

Tests of the concentration of virus present in a soil or tissue therefore consisted simply of extracting a given volume or weight of the material with water and rubbing the extract over the leaves of young hybrid or *Nicotiana glutinosa* plants. Samples of soil for inoculation were usually taken by inserting a sterilized  $\frac{3}{8}$ -inch cork borer into the soil to a depth of 1 inch and ejecting the sample by means of a sterile glass plunger. The sample was placed in a sterilized test tube and 5 cc of water added. After two vigorous shakings, the soil was allowed to settle out for from 2 to 5 minutes and the supernatant liquid poured off into a watch glass and inoculated into the test plants. The average dry weight of soil samples taken by this method was approximately 1 g. When plant parts were used for soil inoculum, the samples were taken from the tissues themselves, or what remained of them following decay. These were necessarily estimated by volume, about 2 to 5 g, according to the tissue, being taken for extraction with 10 cc of water. Original extract used for control inoculations was usually stored at a dilution of 1 to 1 or 1 to 20, but was brought to a final dilution of 1 to 1,000 of the original strength just before inoculation.

Counts of the number of local lesions on the test plants could usually be made on the third or fourth day after inoculation. In the case of the hybrid, these were based on the total number of spots

appearing on the three leaves showing the maximum amount of infection (usually four or five leaves being inoculated on each plant), and in the case of *Nicotiana glutinosa*, on a similar five-leaf count. Lesions numbering less than about 300 were usually counted accurately, while those above 300 were sometimes only estimated, and are then shown in round numbers in the tables. While the plants used in any one series of tests could be selected for uniformity of size and vigor, it was not always possible to secure comparable plants for successive tests made on the same material throughout the year. This factor, together with the natural individual differences in the test plants, introduced considerable variation into the determinations. This was counteracted, in part, by making counts on selected leaves, and in certain cases by using two test plants for each determination and taking the average of the two readings. It is not believed that the method of extraction of the virus from the soil was responsible for much variation in results. The number of shakings, or the time allowed for the soil to settle did not appear greatly to influence the number of lesions obtained. That all the virus was not, of course, removed from the soil by this method could be shown by repeated extractions from the same sample. For the type of information sought in the present investigation, however, namely, the presence or absence of virus or a rough approximation of its concentration, the experimental methods used were considered satisfactory. For more detailed studies, these methods could no doubt readily be modified.

#### EXPERIMENTAL RESULTS

The data presented in the following tables for the most part illustrate representative tests on the various factors under consideration. Slight modifications introduced into successive trials of the same general nature make it impractical to present all the data on which conclusions are based.

##### INFLUENCE OF VARIOUS FACTORS ON THE BEHAVIOR OF THE TOBACCO MOSAIC VIRUS IN THE SOIL

##### LEACHING FROM MOSAIC-INFECTED TISSUES

In planning experiments on the behavior of the tobacco mosaic virus in the soil, it may be assumed that the virus characteristically overwinters in the dead tissues of preceding crops remaining in the field after harvest. It is possible that the primary infection occurring on succeeding crops results entirely from a direct transfer of virus from such plant parts to the new crop. On the other hand, it has been the writers' experience that the tobacco mosaic virus is quickly and easily extracted from dead tissues by water in contact with such material, and it seems likely that a similar condition occurs in the soil itself. Consequently, it may be concluded that soil in the immediate vicinity of virus-carrying tissues contains virus that has leached out from the plant parts during periods of rainfall. How far the virus may leach out and how far it may be carried in soil water must depend upon a number of circumstances. Some simple preliminary experiments have shown that a large part, if not all, of the virus present in dead tissues may eventually be leached out and that it may be transported in this process for considerable distances, and by surface drainage presumably almost indefinitely.

In one experiment, 25 g of Connecticut soil was placed in each of six stoppered Gooch funnels to a depth of about 1 inch. The soil was soaked down with water, and dried infected roots, stubble wood, stalks, sucker stems, and leaf lamina, and living green leaves were placed in separate funnels on the surface of the soil. The plant parts were moistened, and the corked funnels allowed to stand in a warm greenhouse (about 85° F.) to favor rapid decomposition of the tissues. At weekly intervals, 5 cc of water was added to the plant parts and allowed to drain through the soil and out of the funnel, where it was collected and inoculated into hybrid plants. The results presented in table 1 show that considerable quantities of virus were thus leached out from the tissues and were able to pass through the soil. When the soil filtrates no longer yielded infective material it was no longer possible to recover virus from the plant parts themselves by inoculation.

TABLE 1.—*Leaching of tobacco virus 1 through 1 inch of Connecticut soil (25 g) from various decaying tobacco plant tissues on the soil surface, following periodic addition of 5 cc of water*

Period of decomposition of tissues (weeks)	Lesions on 3 leaves of hybrid inoculated with soil leachings from—					
	Dried infected—					Green leaves
	Roots	Stubble wood	Stalks	Sucker stems	Leaf lamina	
	Number	Number	Number	Number	Number	Number
1.....	34	52	235	3	700	13
2.....	10	9	390	26	265	30
3.....	11	3	219	3	240	23
4.....	2	3	12	0	161	6
5.....	0	1	1	0	230	10
6.....	0	1	0	0	161	5
7.....					145	0
9.....					106	
11.....					17	
14.....					2	0

<sup>1</sup> Tissues accidentally dried out.

In another experiment, soil columns 18 inches in height were used in cylindrical copper containers, and virus, added in the form of extract at a dilution of 1 to 100, was found to filter for this distance through soils of various types, though much more readily through sandy than through clay soils. To eliminate the possibility that the virus had followed an uninterrupted path along the walls of the containing vessels, another test was made in which small collecting funnels were inserted into a soil column at different levels in such a way that the filtrate could enter the funnels only after passing directly through the soil for various distances. Inoculation of liquid collected from the funnels showed that virus had passed through the soil for all distances tested, in this case 2, 4, and 6 inches. It seems very likely that leaching of the virus from infected tissues in the field and the mixing of the soil due to preparation of the land for planting may result in a fairly uniform distribution of virus in the soil following heavily infected crops.

From these and other considerations it seems reasonable to assume that the area of soil contaminated through leaching is likely to be a more significant factor in soil infestation than the actual concentration of virus in the dead plant tissues. For this reason, as well as for purposes



of securing an even distribution of virus in the soils to be studied, infestation of soils was usually made with virus extract, though infected plant tissues were also used in several experiments.

#### SOIL TYPE

Earlier tests by Johnson and Ogden (6) had indicated that the tobacco mosaic virus might remain active for a much longer time in some soils than in others. Aeration was suspected as being one factor influencing the longevity of the virus in the soil, and it was suggested that this might be related to the soil type. Consequently a preliminary experiment was planned, according to the methods already described, in which the behavior of the tobacco mosaic virus was studied in soils of different type. Seven representative soils were selected for the purpose (table 2) and placed in duplicate in 2-kg lots in the enamel pans. The soils were inoculated with 200 cc of virus extract diluted 1 to 1 with water, and as a control, 2,000 cc of virus extract at a dilution of 1 to 20 was also placed in each of two pans. One series of pans was allowed to stand in the greenhouse with the covers off to permit free aeration and drying of the soil, whereas the other series remained covered. Both sets were stirred and brought up to weight with water weekly, but the soil repeatedly became relatively dry in the uncovered pans in the intervals between weighings.

Soil samples were taken at various intervals and inoculated to both hybrid and *Nicotiana glutinosa* plants. The hybrid regularly yielded a much higher lesion count than did *N. glutinosa*, and as a result the hybrid was used as test plant in most subsequent experiments. It should be stated that in the present experiment the *N. glutinosa* plants used for inoculation purposes tended to be somewhat older than most suitable for this work, and this fact may be partly responsible for the low counts obtained, especially in some of the earlier inoculations.

TABLE 2.—Relative rate of inactivation of tobacco virus 1 added in the form of extract to different moist soils stored at moderate temperatures in covered and uncovered pans

[Pans brought up to original weight at intervals by addition of water]

Soil <sup>1</sup>	Pan cover	Average weekly loss of water	Lesions on test plant after indicated period									
			On hybrid			On <i>Nicotiana glutinosa</i>						
			0 week <sup>2</sup>	1 week	2 weeks	0 week <sup>2</sup>	1 week	4 weeks	7 weeks	12 weeks		
		Grams	No.	No.	No.	No.	No.	No.	No.	No.		
Wisconsin (B).....	On.....	6	600	600	500	250	65	42	70	68		
	Off.....	93	600	300	76	200	27	0	0	0		
South Carolina (2).....	On.....	12	700	600	400	200	60	120	6	25		
	Off.....	167	550	350	84	175	17	0	0	0		
Florida.....	On.....	8	500	500	500	200	44	83	7	18		
	Off.....	170	350	200	300	225	3	0	0	0		
Ohio.....	On.....	4	450	125	135	85	6	12	3	15		
	Off.....	166	350	102	42	49	1	0	0	0		
Pennsylvania.....	On.....	5	450	80	57	25	2	31	1	1		
	Off.....	194	350	0	0	55	0	0	0	0		
Connecticut.....	On.....	8	275	315	300	94	14	21	12	14		
	Off.....	163	250	60	8	45	6	1	0	0		
Tennessee (C).....	On.....	5	97	36	36	2	1	6	2	5		
	Off.....	149	200	0	1	27	0	0	0	0		
Virus extract (stored at 1 to 20 dilution).....	On.....	14	275	600	500	175	18	0	0	0		
	Off.....	396	194	300	300	95	42	0	0	0		

<sup>1</sup> See explanation of letters and numerals on p 272.

<sup>2</sup> Immediately after infestation with the virus

Considerable variation will be noted in the number of lesions obtained in the duplicate inoculations from some of the soils made immediately after infestation with the virus (table 2). In spite of this variation, there is still some indication that the soils themselves may vary significantly in their immediate effect upon the virus. The reason for this is not clear, though at the time it was suspected that differences in adsorptive capacity of the soils might be partly responsible. On the other hand, it is possible that the differences noted might be due merely to the difficulty of securing perfectly uniform distribution of the virus in the soil. However, further evidence of a similar nature obtained in other experiments, to be discussed later, points to the existence of inherent differences in certain soils with respect to their immediate effect upon the virus.

Subsequent inoculations from the different soils (table 2) showed a gradual inactivation of the virus in the covered pans in the absence of drying and in reduced aeration. More striking was the relatively rapid and complete inactivation that occurred in the uncovered pans, where the soil was exposed to drying and more complete aeration. There was also some indication that the rate of inactivation was slower in certain soils, e. g. Wisconsin (B) soil, than in others, under the same conditions. Such differential behavior, however, will be more clearly demonstrated in later experiments discussed in connection with the aging or persistence of the virus in different soils, (see tables 17, 18, and 19). The preliminary experiment suggests that several different major factors need to be considered as playing a possible part in the inactivation of the tobacco mosaic virus under natural conditions, namely, adsorption, aeration, desiccation, and possibly microbial action. In subsequent experiments, attention has been given to these and other factors individually, while attempts have been made to maintain other conditions as constant as possible.

#### ADSORPTION

It is well known that charcoal and certain other substances of both organic and inorganic nature have a peculiar affinity for the tobacco mosaic virus, resulting in its partial or complete inactivation. This takes place presumably through adsorption of virus particles. It seems not unlikely that soils, varying greatly as they do in physical and chemical characters, might also exercise some adsorptive capacity for the virus. Indications that this might be so have already been noted in the marked variation obtained in initial lesion counts when virus extract was added to different soils (table 2). Accordingly, a more detailed comparison was made of the adsorptive capacity for the tobacco mosaic virus of 26 different soils, varying from light sands to heavy clays, with that of peat, charcoal, kaolin, kieselguhr, talcum powder, and glass powder.

Since it seemed likely that the adsorptive power of the soil would be influenced by its physical structure, more particularly by the percentage of colloidal material (clay) present, a physical analysis of the soils was made by the Bouyoucos hydrometer method (1). The percentage of total colloids, silt, and sand in the different soils is shown in table 3, together with the pH value of each soil as determined by the electrical method, and the results of three tests of the adsorptive capacity of different soils for the virus. In the first of these tests,

10 cc of concentrated virus extract was added to 100 g of air-dry soil and allowed to stand for 24 hours. Extracts of soil samples were then made in the usual way and inoculated into hybrid plants. In the second and third tests, 10 cc of virus extract at a 1 to 500 dilution was added to 10 g of soil, and allowed to stand for about 1 hour. Ten cubic centimeters of water was then added and the suspension filtered through filter paper. Still other tests were made in an attempt to compare the adsorptive capacity of certain soils with that of other substances of known high adsorptive power. Only selected results are presented in table 4, which shows a comparison of filtrates from New York and South Carolina (1) soils, sand, talcum powder, and animal charcoal, following the addition of equal amounts of virus extract to equal volumes of soil or other substances.

TABLE 3.—*Type, physical analysis, and hydrogen-ion concentration of the different soils used in the investigation, together with a comparison of the adsorptive capacity of different soils for tobacco virus 1 added in the form of extract*

Soil <sup>1</sup>	Type	Col- loids	Silt	Sand	pH (elec- trical meth- od)	Lesions on 3 leaves of hybrid from soil filtrate soon after virus was added in experiment no.—		
						1	2	3
		Pct	Pct	Pct		No.	No	No
Wisconsin (R).....	Superior red clay.....	50 4	13 2	36 4	5 4	175	135	.....
West Virginia.....	Huntington silt loam.....	48 6	29 0	22 4	5 5	250	80	200
Kentucky.....	Hagerstown clay (?).....	44 4	33 0	22 6	7 4	105	.....	.....
Tennessee (Greenville) (C).....	.....	39 0	24 4	36 6	8 1	82	180	535
Pennsylvania.....	Hagerstown loam.....	37 8	34 6	27 8	7 9	109	138	251
Tennessee (S).....	Clarksville silt loam.....	36 2	43 2	20 6	7 1	304	156	390
Ohio.....	Miami clay loam.....	35 2	40 0	24 8	7 1	175	260	600
New York (Elmira).....	.....	33 4	22 8	43 8	6 0	200	293	96
Wisconsin (W).....	Carrington silt loam.....	31 8	34 2	34 0	5 8	.....	.....	176
Virginia.....	Cecil red clay (?).....	30 2	24 6	45 2	5 0	375	.....	.....
Wisconsin (S).....	Miami silt loam.....	29 8	43 2	27 0	8 2	162	112	.....
Greenhouse.....	Compost.....	28 0	26 4	45 6	7 5	98	.....	.....
Wisconsin (E).....	Miami silt loam (?).....	27 8	33 0	39 2	6 1	.....	.....	.....
Wisconsin (G).....	Knox silt loam.....	20 8	44 2	35 0	4 8	.....	.....	.....
Massachusetts.....	Agawam sandy loam.....	17 8	19 0	63 2	5 5	150	162	.....
Wisconsin (C).....	Waukesha silt loam.....	17 4	34 2	48 4	8 5	300	105	294
Connecticut.....	Merrimac sandy loam.....	14 6	15 6	69 8	5 5	665	106	259
Wisconsin (L).....	Sparta sand.....	12 4	6 2	81 4	5 2	.....	.....	146
North Carolina.....	Durham sandy loam.....	12 0	5 2	82 8	6 1	325	120	119
Maryland.....	Collington fine sandy loam.....	9 4	7 8	82 8	5 7	.....	68	322
Florida.....	Orangeburg sandy loam.....	9 2	2 0	88 8	5 8	500	95	.....
Georgia.....	Norfolk sandy loam.....	9 0	7 0	84 0	5 3	350	.....	.....
South Carolina (3).....	do.....	8 0	2 4	89 6	7 1	625	.....	.....
Wisconsin (Brooklyn) (B).....	.....	7 2	4 0	88 8	6 0	162	118	230
South Carolina (1).....	Norfolk sandy loam.....	7 0	4 0	89 0	7 8	375	138	167
South Carolina (2).....	do.....	6 6	5 0	88 4	5 7	105	.....	347
Peat.....	Peat.....	?	?	?	7 1	375	.....	.....

<sup>1</sup> See explanation of letters and numerals on p 272

TABLE 4.—*Comparison of the adsorptive capacity of equal volumes of soils and other materials for tobacco virus 1, used in the form of extract diluted to 1 to 1,000*

Soil or material <sup>1</sup>	Lesions on 3 leaves of hybrid in experiment no.—		
	1	2	3
	Number	Number	Number
New York.....	293	317	107
South Carolina (1).....	138	61	93
Sand.....	.....	110	90
Talcum powder.....	0	0	0
Animal charcoal.....	0	0	0
Virus extract only (1 to 1,000 dilution).....	87	78	80

<sup>1</sup> See explanation of numeral on p 272

In these preliminary trials there were again indications of some immediate effect of the soil on the virus, though there appeared to be little correlation with the physical character of the soil, or with the hydrogen-ion concentration (table 3). On the other hand, none of the soils approached the high degree of inactivation exhibited by charcoal and other finely powdered substances, such as talcum, glass powder, kaolin, etc. During these and other trials, it became evident, however, that consistent and significant differences existed between certain soils with respect to their immediate effect upon the virus. This is more clearly illustrated in table 5, which shows a comparison of the immediate effect upon the virus of five selected soils when wet, air-dry, and oven-dry. In this test, 15 cc of 1 to 500 virus extract was added to about 10 g of soil, followed later by 15 cc of water, making a final dilution of virus of 1 to 1,000. The liquid was then filtered through filter paper and inoculated to hybrid plants. The difference between the New York and West Virginia soils is clearly significant, and apparently too great to be accounted for on the basis of differences in physical structure. On the other hand, it will be noted that the original moisture content of the soil had no significant effect on the amount of inactivation of the virus when added in the form of liquid extract.

It seems likely that some other, as yet undetermined, factor is involved in this rapid partial inactivation of the virus in the soil. For the present, it has not been thought necessary to investigate this particular phenomenon further. It will be shown later that this immediate partial inactivation bears no direct relation to the subsequent rate of inactivation of the virus following prolonged association with the soil. For practical purposes relating to mosaic control, it seems legitimate to regard all soils as essentially similar so far as immediate inactivation or possible adsorption of the virus is concerned. However, the physical properties of the soil may influence certain other factors, such as rate of dessication, which in turn may be indirectly related to adsorption phenomena; consequently adsorption may still be significant in relation to the ultimate rate of inactivation of virus in the soil.

TABLE 5.—*Comparison of amount of adsorption of tobacco virus 1 added in the form of extract to different soils of varying types and reactions and of different moisture content*

Soil <sup>1</sup>	Total col- loids	pH	Lesions on 3 leaves of hybrid from virus added to—		
			Oven-dry soil	Air-dry soil	Wet soil
	Percent		Number	Number	Number
Wisconsin (C).....	17.4	8.5	130	202	144
New York.....	33.4	6.0	225	184	181
Tennessee (C).....	39.0	8.1	55	67	106
Wisconsin (R).....	50.4	5.4	84	56	50
West Virginia.....	48.6	5.5	30	27	19
Virus extract only (1 to 1,000 dilution).....				126	

<sup>1</sup> See explanation of letters on p. 272

The untreated virus extract itself often produced fewer lesions on the hydrid than after addition to certain soils (tables 4 and 5). This situation has been observed repeatedly and is believed to be a consequence of one or both of two circumstances, namely, the reduction or breaking up of clumps of virus particles through shaking with the soil, or the result of the abrasive action of soil particles during the process of inoculation.

#### SOIL REACTION

Although the tobacco mosaic virus has recently been shown to be active in extract over a wide range of hydrogen-ion concentrations (8), it is conceivable that the reaction of the soil might have some influence on the immediate or subsequent inactivation of virus added thereto. The pH value of all soils used in this investigation was therefore determined by the electrical method (table 3) and checked by the Truog method, the latter giving slightly lower readings. The soils ranged from very acid (pH 4.8) to quite alkaline (pH 8.5). As shown in tables 3 and 5, there was no significant correlation, however, between the hydrogen-ion concentration of the soils and any immediate effect upon the virus. Moreover, although, as will be shown later, the subsequent rate of inactivation of the virus was found to vary considerably in certain soils, again there was no consistent relation between inactivation and the soil reaction.

A further test was made with two very acid soils obtained from western Wisconsin, in the vicinity of Gays Mills (Wisconsin (G) soil) and La Valle (Wisconsin (L) soil). Two kilograms of each soil was placed in each of four pans, and lime was added to each series in the proportion of 0, 10, 20, and 30 g per pan, respectively. The lime was thoroughly mixed into each lot of soil, and the lots were inoculated with equal amounts of virus extract. The soils were brought up to, and maintained at, the same moisture content by means of weekly weighings. Soil samples were taken at intervals over a period of 17 weeks and inoculated into *Nicotiana glutinosa* plants (table 6). Although the rate of inactivation of the virus appears to have been somewhat higher in the most acid lot of the La Valle soil, this was not so in the case of the Gays Mills soil, which was even more acid. Everything considered, it appears that the reaction of the soils did not exert any significant influence on the rate of inactivation.

TABLE 6.—Rate of inactivation of tobacco virus 1 added in the form of extract to two different soils maintained at various hydrogen-ion concentrations by adding lime

[Soil kept in a moist condition and stored at moderate temperatures]

Soil <sup>1</sup>	Amount of lime added per pan of soil	pH	Lesions on 5 leaves of <i>Nicotiana glutinosa</i> after—								Average number of lesions
			0 week <sup>2</sup>	1 week	3 weeks	5 weeks	8 weeks	10 weeks	15 weeks	17 weeks	
	Grams		No.	No.	No.	No.	No.	No.	No.	No.	
Wisconsin (L).....	0	4.89	40	52	79	103	17	46	8	6	44
	10	7.58	55	62	278	197	18	55	29	8	88
	20	8.25	131	71	184	151	10	40	34	10	79
	30	8.64	85	153	240	132	25	141	28	37	105
Wisconsin (G).....	0	4.80	51	59	247	203	77	170	75	88	121
	10	7.43	22	43	147	101	43	96	21	15	54
	20	8.30	55	92	128	190	3	14	24	5	64
	30	8.33	139	71	286	183	10	70	9	13	98
Virus extract (1-1,000 dilution).....			18	15	176	136	13	183	-----	-----	90

<sup>1</sup> See explanation of letters on p. 272

<sup>2</sup> See footnote 2, table 2.

## SOIL-MOISTURE CONTENT

Perhaps the most variable factor to which soils are exposed is that of moisture content. It was early suspected that this factor would have some effect on the longevity of the tobacco mosaic virus in the soil through its influence on microbial activity. It is well known that this virus will persist for years in dried plant tissues, but that in the presence of moisture and air, which permit microbial action, the virus will gradually become inactivated (6). In the present connection, however, a quite different moisture relation is emphasized, namely, the immediate effect of dessication of the soil upon the virus.

This effect was first observed in a preliminary experiment designed to test the relation of the soil-moisture content to the rate of inactivation of virus added in the form of extract to the soil. Two widely different soils were chosen, one a South Carolina sandy loam (South Carolina (1)) and the other a black Wisconsin silt loam (Wisconsin (W)). Determinations were made of the water-holding capacity of each soil and 2-kg lots were then made up to, and maintained at, different moisture contents by addition of virus extract and water, as shown in table 7. The two lots planned as controls were kept in an air-dry condition and were consequently air-dried as soon as possible after addition of the virus extract. Inoculations made the following morning showed that the virus was already almost completely inactivated in both dried soils, although present in considerable quantity in all moist soils. Subsequent tests made over a period of 17 weeks showed a gradual decline in virus concentration in the moist soils and continued, almost complete inactivation in the air-dry lots. While there is some suggestion of variation in the rate of inactivation at different moisture contents apart from drying, within normal limits for plant growth, at least, this factor does not appear to be of major importance.

TABLE 7.—Rate of inactivation of tobacco virus 1 added in the form of extract to two different soils stored at moderate temperatures and maintained at various moisture contents

Soil <sup>1</sup>	Moisture content as percentage of saturation	Lesions on 5 leaves of <i>Nicotiana glutinosa</i> after—								Average number of lesions
		0 week <sup>2</sup>	1 week	3 weeks	5 weeks	8 weeks	10 weeks	15 weeks	17 weeks	
	Percent	Number	Number	Number	Number	Number	Number	Number	Number	
South Carolina (1)-----	100	137	333	196	104	163	175	87	33	153
	75	159	180	252	190	108	66	43	14	126
	50	153	328	284	160	205	58	30	14	154
	25	167	233	334	270	504	92	31	25	207
	<sup>3</sup> 0	5	14	7	24	5	7	4	0	8
Wisconsin (W)-----	100	88	36	80	291	70	14	2	5	73
	75	63	84	313	330	171	125	85	23	149
	50	66	130	91	310	193	147	66	21	128
	25	105	31	65	130	49	61	5	13	57
Virus extract (at 1-1,000 dilution)-----	<sup>2</sup> 0	1	0	0	1	0	1	0	0	0
		18	15	176	136	13	188			90

<sup>1</sup> See explanation of letter and numeral on p. 272.

<sup>2</sup> See footnote 2, table 2.

<sup>3</sup> Air-dry.

Various other experiments have been made to test this striking influence of drying on the inactivation of the virus in the soil. All have yielded results of a similar nature. Thus, exposure of red-clay soil (Wisconsin (R)) to drying for as short a time as 1 hour following

addition of virus extract reduced the virus content to at least one-fifteenth of the original amount, as judged by inoculations into hybrid plants. In sand the rate and percentage of reduction were least, whereas in other soils of intermediate type they were intermediate in this respect. This behavior suggests the possibility of some adsorption phenomenon coming into play during drying. It does not, however, eliminate the possibility that aeration plays some part in this rapid inactivation; consequently, further experiments were planned to test this relationship.

#### AERATION

The inactivating influence of oxygen on tobacco mosaic virus extract has previously been reported (3), and its relation to inactivation of the virus in the soil has been suggested (6). In soil studies, however, the marked effect of dessication on the virus necessitated the planning of more carefully conducted tests. Thus, various attempts have been made to separate the factors of aeration and dessication in different ways.

In one experiment, a soil rotator was devised, permitting constant stirring of the soil within a glass tube 4 inches in diameter, in which the amount of oxygen and atmospheric humidity could be approximately controlled by drawing air, conditioned as desired, through the rotator. Equally satisfactory results were obtained by placing smaller quantities (5 g) of soils in desiccators over either water or calcium chloride or sulphuric acid, and removing the oxygen when desired by means of pyrogallol potassium hydroxide or by displacement with illuminating gas (table 8). In a third experiment, 2-kg lots of soil were used in enamel pans either covered or uncovered, with daily regulation of soil moisture and stirring of the soil, where desired, over a period of 4 weeks (table 9). All methods yielded similar results. It became clear from these trials that, while the presence of oxygen may cause a definite increase in the rate of inactivation of virus extract in the soil over considerable periods, it can in no way be held responsible for the immediate or rapid inactivation of the virus resulting from exposure of the soil to air-drying. It may be concluded that it is the actual dessication of the soil that causes the rapid inactivation of virus extract present therein. It must not be inferred, however, that a similar behavior results on the part of virus present in organized plant tissues in desiccated soils. This situation will be discussed in detail later.

TABLE 8.—Comparison of the influence of oxygen and of desiccation on the inactivation of tobacco virus 1 added in the form of extract to three different soils (desiccator method)

Condition of atmosphere <sup>1</sup>		Regulation <sup>2</sup> by—	Lesions on 3 leaves of hybrid from soil from—		
Oxygen	Moisture		Connecticut <sup>3</sup>	Georgia	Virginia
			<i>Number</i>	<i>Number</i>	<i>Number</i>
+	+	Water.....	400	67	86
+	—	Calcium chloride.....	122	0	0
+	—	Sulphuric acid.....	51	0	0
—	+	Illuminating gas + water.....	400	213	60
—	+	Pyrogallol acid — potassium hydroxide + water.....	181	122	55
—	—	Illuminating gas + calcium chloride.....	38	0	0
Control (untreated infested moist soil).....			660	75	91

<sup>1</sup> + present, — absent.

<sup>2</sup> For 48 hours.

<sup>3</sup> This soil was not completely desiccated.

TABLE 9.—*Comparison of the influence of aeration and drying, through regulation of air and water supply by periodic stirring and watering, on the inactivation of tobacco virus 1 in two soils in covered and uncovered pans*

[Virus added in the form of extract to 2-kg lots of soil]

Soil	Aeration	Water supply	Lesions on three leaves of hybrid after—			
			1 day	7 day	14 days	28 days
			<i>Number</i>	<i>Number</i>	<i>Number</i>	<i>Number</i>
Ohio	Low	High	440	74	124	372
	Medium	do.	135	115	45	105
	High	Medium	154	163	46	18
	do.	Low	372	105	18	1
North Carolina	Low	High	207	140	30	79
	Medium	do.	221	150	12	10
	High	Medium	365	57	1	0
	do.	Low	364	6	0	0

## SOIL TEMPERATURE

Under natural conditions the soil is exposed to wide fluctuations in temperature, which might be expected to have some influence on the virus content of the soil. On account of the high thermal death point of the tobacco mosaic virus, however, little direct effect of high soil temperatures is to be expected. Such temperatures are likely to be concerned chiefly with the rate of microbial activity in the soil and the resultant decomposition of infected plant tissues. Judging from the behavior of the tobacco mosaic virus in extract at different temperatures, it might be anticipated that low soil temperatures would act in a preservative manner, preventing decay and generally prolonging the life of the virus. That this is not necessarily so will be shown in the following experiments, in which freezing temperatures in particular were found to cause relatively rapid inactivation of virus extract in the soil.

A preliminary experiment was planned to test the relative rate of inactivation of the virus in two soils (Wisconsin (S) and Maryland soils) at a series of temperatures ranging from approximately 0° to 40° C. (table 10). Virus was added to the soils in the form of extract and the soils were stored in a moist condition in 2-kg lots in covered pans, placed in incubators kept at constant temperature. Daily readings were made of the temperature of each incubator and were found to vary very little from week to week. The mean of the daily readings for each incubator is given in table 10. As a control, virus extract diluted to 1 to 20 with water was stored in small stoppered bottles, one of which was placed in each chamber.

At temperatures of approximately 5° to 30° C., significant differences in the rate of inactivation of the virus over a number of weeks were found to be either lacking or very slow to develop. At 40° C., inactivation was distinctly more rapid in both soils, though not so evident in the extract. The most striking effect, however, occurred at approximately 0° C., where the decline in virus content of both soils was even more marked than at 40° C., although the virus extract stored in bottles at that temperature showed no comparable change. It seems likely that the more rapid inactivation at higher temperatures was due to the rate or type of microbial activity. The inactivation at 0° C., however, was more obscure and necessitated additional tests at low temperatures.



TABLE 10.—*Influence of temperature on the rate of inactivation of tobacco virus 1 added in the form of extract to two different moist soils*

Extract in—	Mean tem- per- ature	Lesions on 3 leaves of hybrid after—											Ave- rage num- ber of lesions
		0 weeks (1)	1 week	2 weeks	5 weeks	7 weeks	8 weeks	10 weeks	12 weeks	14 weeks	16 weeks		
		° C	Num- ber	Num- ber	Num- ber	Num- ber	Num- ber	Num- ber	Num- ber	Num- ber	Num- ber		
Wisconsin (S) soil :	(-0.4	406	85	47	28	42	9	16	4	-----	-----	80	
	4.8	345	570	279	277	400	300	-----	-----	-----	-----	362	
	8.3	722	703	406	351	399	560	156	58	154	161	367	
	15.8	425	792	127	494	283	402	-----	-----	-----	-----	420	
	19.6	-----	446	84	196	419	348	20	135	177	11	204	
	24.2	543	354	174	84	150	201	-----	-----	-----	-----	251	
	29.5	415	215	219	120	157	210	70	14	-----	-----	177	
	40.3	540	423	10	25	3	34	8	8	-----	-----	131	
	(-2.0	366	192	63	76	116	50	7	10	-----	-----	110	
	4.8	365	660	243	1,250	606	444	-----	-----	-----	-----	595	
Maryland soil.	8.3	-----	703	275	866	712	630	710	830	520	590	648	
	15.8	296	341	394	953	272	490	-----	-----	-----	-----	458	
	19.6	550	407	169	780	826	593	664	670	441	398	550	
	24.2	562	457	244	696	745	456	-----	-----	-----	-----	527	
	29.6	554	340	307	634	560	440	248	326	100	115	362	
	40.3	490	434	98	215	90	110	43	50	12	4	155	
	(-0.4	295	53	188	363	170	311	258	247	-----	240	236	
	4.8	-----	114	114	290	111	232	-----	-----	-----	-----	172	
	8.3	-----	137	73	179	279	343	179	49	-----	30	159	
	15.8	-----	314	86	171	320	168	-----	-----	-----	-----	212	
Water (stored at dilution of 1 to 20, tested at 1 to 1,000).	19.6	-----	308	110	256	92	285	365	158	-----	326	237	
	24.2	-----	254	80	194	369	215	-----	-----	-----	-----	222	
	29.6	-----	467	135	129	38	78	46	1	-----	1	112	
	40.3	-----	72	58	172	64	194	223	202	-----	119	138	

1 See footnote 2, table 2.

2 See explanation of letter on p 272

In a second and similar experiment the same two soils were tested at three different temperatures below freezing (approximately  $-1^{\circ}$ ,  $-10^{\circ}$ , and  $-21^{\circ}$  C.) in comparison with controls stored at approximately  $8^{\circ}$  C. (table 11). All soils were in a moist condition at the beginning of the experiment and were kept up to weight by the regular addition of water, where required. The freezing of the soil, regardless of the actual temperature reached, caused relatively rapid inactivation of the virus present therein, as compared with the controls. The frozen soils appeared to be in a very dry condition, although still maintaining their original weight. This was believed to be due to the low temperature causing the water to crystallize out of the soil in the form of ice. In subsequent experiments, therefore, various soils either partly or fully saturated with water were tested at temperatures below and above freezing, with similar results (table 12). It is evident that freezing causes relatively rapid inactivation of virus present in the form of extract in the soil, presumably as a result of desiccation of the soil and not as a direct effect of the low temperatures, since there is little effect on the virus itself at these temperatures when tested either in extract alone (tables 10, 11) or in plant tissues in the soil (tables 14, 15). It seems certain, therefore, that, under field conditions, such virus as leaches into the soil prior to the freezing of the soil must become largely, if not entirely, inactivated. Hence it may reasonably be assumed that in cooler climates any free virus present in the soil at the time of field planting has leached into it during the spring, whereas in tropical or subtropical climates this particular form of inactivation must be normally lacking.

TABLE 11.—*Influence of freezing temperatures on the rate of inactivation of tobacco virus 1 added in the form of extract to two different moist soils*

Extract in—	Mean temperature	Lesions on 3 leaves of hybrid after—						Average number of lesions after first week
		0 week <sup>1</sup>	1 week	3 weeks	5 weeks	7 weeks	9 weeks	
	(° C.)	No.	No.	No.	No.	No.	No.	
Wisconsin (S) soil <sup>2</sup> .....	-1.4	201	64	22	7	8	-----	25
	-8.9	300	92	11	2	3	-----	27
	-20.6	402	15	24	2	0	-----	10
	+8.5	580	156	58	154	161	-----	132
	-1.4	456	340	265	25	27	-----	132
Maryland soil.....	-9.0	444	380	126	78	3	4	178
	-20.6	490	870	224	106	1	5	241
	+8.5	630	710	830	520	590	98	550
Water (stored at dilution of 1 to 20, tested at 1 to 1,000).....	-20.6	232	430	282	-----	260	91	266

<sup>1</sup> See footnote 2, table 2<sup>2</sup> See explanation of letter on p 272TABLE 12.—*Influence of freezing temperatures on the rate of inactivation of tobacco virus 1 added in the form of extract to different soils either partially or fully saturated*

## SOIL SEMISATURATED WITH WATER

Extract in—	Temperature		Lesions on 3 leaves of hybrid after—						Average number of lesions after first week
	Mean	Range	0 week <sup>1</sup>	1 week	2 weeks	4 weeks	6 weeks	8 weeks	
	(° C.)	(° C.)	No.	No.	No.	No.	No.	No.	
Pennsylvania soil.....	-1.7	-1.2 to -2.1	1,070	301	-----	390	9	7	177
	15.7	15.1 to 16.2	923	835	-----	465	50	106	364
Georgia soil.....	-3.2	-2.9 to -4.2	880	636	-----	256	52	17	240
	15.7	15.1 to 16.2	893	670	-----	730	350	560	577
Full strength extract (tested at dilution of 1 to 1,000).	4.8	4.3 to 5.6	-----	-----	-----	237	-----	302	269

## SOIL SATURATED WITH WATER

Florida soil.....	-1.3	-0.9 to -1.7	200	-----	39	16	-----	-----	27
	-4	7 to -19	135	-----	19	1	-----	-----	10
	16.0	15.8 to 16.4	310	-----	166	104	-----	-----	135
New York soil.....	-3.5	-3.1 to -4.3	193	-----	62	27	-----	-----	44
	-4	7 to -19	72	-----	52	1	-----	-----	26
	16.0	15.8 to 16.4	55	-----	218	94	-----	-----	156

<sup>1</sup> See footnote 2, table 2<sup>2</sup> Soil outdoors (Feb. 4 to Mar. 4).

## MICROBIAL ACTION

The extreme longevity of the tobacco mosaic virus in bottled liquid extract, without preservatives, and in dry plant tissues is an outstanding characteristic of this virus. The fact that the addition of preservatives prolongs the life of the virus in extract suggests that such slow inactivation as occurs under these conditions may be due partly to decomposition of plant materials through microbial activity, which may be expected to be retarded by the limited oxygen supply and by the toxic effect of the products of decay. Inactivation of the virus is more rapid in moist plant tissues than in bottled extract, perhaps on account of a more plentiful supply of oxygen favorable for microbial growth and also of the supply of cellulose and other sub-

stances available as a medium for microbial, particularly fungous, development. It has repeatedly been shown in this and other investigations that virus extract becomes inactivated only very gradually in the soil at moderate temperatures, and, as indicated in an earlier paper (6), the virus is inactivated even more slowly, if at all, when soil and virus are brought together under aseptic conditions. This suggests that decomposition by itself may result in inactivation.

Although it is believed that much of the virus naturally present in the soil exists in the form of extract leached from infected plant tissues, the writers' experiments indicate that virus present in the soil in this form is subject to more vigorous types of inactivation than that remaining in plant tissues. Thus, tests of the persistence of virus in infected plant parts incorporated into the soil have shown that freezing does not cause inactivation of the virus present in the tissues (tables 14 and 15), nor does desiccation of undecayed tissues (table 14). According to the writers' observations, the gradual loss of virus from dead plant parts below the soil surface, under natural conditions, must result largely either from leaching out of the virus or from decomposition, or, more probably, from a combination of both factors.

The rate of decomposition of the tissues and of inactivation of the virus probably depends on a number of factors, such as the size and type of the plant parts (roots, stalks, leaves, etc.) and on the soil temperature, moisture, and air supply, as well as on the soil character and types of organisms present. In the present investigation, attempts have been made to compare the extent and rate of such decomposition under different conditions by exposing various types of virus-infected tissue, such as roots, stalks, and leaves to decay at different temperatures in a very moist atmosphere in the absence of soil (table 13), and under winter conditions in the presence of soil (tables 14 and 15).

In the first experiment, dried roots, stubble wood, stalks, and leaf lamina, fresh green leaves, and virus extract were placed in separate glass beakers set on a layer of moist sand in each of five enamel pans. Four pans were kept closed, the fifth open (table 13). Both plant parts and sand were kept very moist by frequent addition of water. The pans were placed under different temperature conditions, as shown in table 13, and inoculations were made at intervals by the usual method from samples of the different materials. As might be expected, decay of the tissues proceeded at a much higher rate at the higher temperatures than at the lower, and the rate of inactivation of the virus in the tissues appeared to be correlated with the rate of decomposition. Thus, at a temperature of 2° to 3° C., the virus content of the different plant parts remained very high, even after 20 weeks, while at the highest temperature, about 39° to 41° C., at which decay was rapid, the virus content diminished rapidly. It was thought that virus present in the woody tissue of the large roots and base of the stalk might survive longer than that present in the more succulent leaf or sucker tissue. It was found difficult, however, to obtain strictly comparable data on this point, owing either to wide differences in concentration of the virus present in the tissues or to difficulties of extraction. Nevertheless, the results tend to indicate that the stubble and roots of the crop are no more likely to preserve the virus, by virtue of their size and composition, than is the leaf tissue under the same environment.

TABLE 13.—Rate of inactivation of tobacco virus 1 in various tobacco plant tissues allowed to decay naturally at different temperatures in the absence of soil

Temperature range (° C ) and how kept	Number of lesions on 3 leaves of hybrid after indicated period resulting from virus from—																								
	Roots												Dried leaf lamina												
	0 1	2 weeks	3 weeks	4 weeks	7 weeks	9 weeks	12 weeks	16 weeks	20 weeks	24 weeks	0 1	2 weeks	3 weeks	4 weeks	7 weeks	9 weeks	12 weeks	16 weeks	20 weeks	24 weeks					
2 to 3 (refrigerator).....	82	---	146	122	370	88	360	170	390	76	157	---	27	275	11	227	650	250	800	550	---	---	---	---	---
—30 to 18 (outdoors).....	---	75	23	88	54	13	47	94	0	---	---	36	185	75	68	126	45	85	400	600	---	---	---	---	---
16 to 27 (indoors, pan closed).....	---	202	20	8	14	25	9	---	---	---	---	420	850	360	150	18	82	---	13	21	---	---	---	---	---
16 to 27 (indoors, pan open).....	---	---	36	7	0	1	---	---	---	---	---	---	76	75	180	189	186	---	---	---	---	---	---	---	---
39 to 41 (incubator).....	---	7	0	0	1	0	---	---	---	---	---	258	76	34	3	25	---	---	---	---	---	---	---	---	---
	Stubble wood												Fresh green leaves												
2 to 3 (refrigerator).....	41	---	58	74	293	161	103	180	420	240	765	---	725	580	194	51	425	325	310	600	---	---	---	---	---
—30 to 18 (outdoors).....	---	21	3	0	12	3	26	38	---	0	---	480	700	800	64	9	70	171	235	132	---	---	---	---	---
16 to 27 (indoors, pan closed).....	---	252	3	133	68	21	---	---	---	---	---	192	92	118	235	64	37	---	---	---	---	---	---	---	---
16 to 27 (indoors, pan open).....	---	---	117	34	64	4	---	---	---	---	---	---	102	206	51	16	0	---	---	---	---	---	---	---	---
39 to 41 (incubator).....	---	92	6	2	0	0	---	---	---	---	---	209	234	320	66	3	---	---	---	---	---	---	---	---	---
	Stalks												Extract (stored at 1 to 10 dilution)												
2 to 3 (refrigerator).....	53	---	3	93	123	152	574	230	440	409	387	---	258	71	85	75	210	96	---	---	---	---	---	---	---
—30 to 18 (outdoors).....	---	120	13	45	28	4	59	65	4	---	---	290	103	60	143	84	136	144	192	135	---	---	---	---	---
16 to 27 (indoors, pan closed).....	---	260	51	21	1	21	---	---	---	---	---	296	65	84	108	2	5	---	2	10	2	36	---	---	---
16 to 27 (indoors, pan open).....	---	---	22	6	2	0	0	---	---	---	---	---	21	8	0	0	0	---	---	---	---	---	---	---	---
39 to 41 (incubator).....	---	3	1	6	0	0	---	---	---	---	---	0	7	0	0	6	---	---	---	---	---	---	---	---	---

<sup>1</sup> Immediately after infection with the virus.<sup>2</sup> Virus not diluted further for inoculation.

TABLE 14.—Rate of inactivation of tobacco virus 1 in various tobacco plant tissues placed in 2 moist soils and exposed to winter conditions in Wisconsin

[Wisconsin (S) soil (pH 8.2) and Wisconsin (L) soil (pH 5.2) used;<sup>1</sup> experiment begun Sept. 27, 1934]

Date	Minimum temperature over preceding period	Lesions on 5 leaves of <i>Nicotiana glutinosa</i> from—												Extract (stored at 1 to 1 dilution, tested at 1 to 1,000)
		Plant parts in 2 different moist soils <sup>2</sup>						Plant parts stored dry in warm room without soil						
		Roots	Stubble wood	Stalks	Sucker stems	Leaf lamina	Extract	Roots	Stubble wood	Stalks	Sucker stems	Leaf lamina		
1934	° F.	No.	No.	No	No.	No.	No.	No.	No	No.	No.	No	No	
Sept. 27	37	74	50	92	39	46	50	54	35	64	3	---	---	
Oct. 4	45	136	67	376	50	60	78	221	485	16	148	---	---	
Oct. 11	28	7	9	14	16	98	64	687	744	199	820	1, 270	---	
Nov. 1	27	35	78	151	4	325	170	265	508	350	520	900	---	
Nov. 8	26	174	131	111	106	178	54	---	---	---	---	---	---	
Nov. 22	26	174	131	111	106	178	54	---	---	---	---	---	---	
Dec. 19	4	17	6	20	4	177	2	246	312	54	285	554	---	
1935														
Jan. 16	—8	16	2	5	3	58	0	---	---	---	---	---	---	
Feb. 14 <sup>3</sup>	—22	79	2	20	6	130	1	---	---	---	---	---	---	
Mar. 14 <sup>3</sup>	—3	11	3	76	8	151	1	---	---	---	---	---	---	
Apr. 9 <sup>3</sup>	19	88	15	71	39	84	0	---	---	---	---	---	---	
May 9 <sup>3</sup>	21	8	2	5	2	140	0	---	---	---	---	---	---	
June 9 <sup>3</sup>	38	41	1	7	9	46	0	46	48	16	156	1, 250	---	
Total	-----	686	366	948	286	1, 591	531	1, 519	2, 132	699	1, 932	4, 609	756	
Average	-----	57	30	79	24	122	41	253	355	116	322	922	151	

<sup>1</sup> See explanation of letters on p. 272.<sup>2</sup> Figures represent average of readings from 2 different soils.<sup>3</sup> Inoculations made to 3 leaves of hybrid plants.

TABLE 15.—*Rate of inactivation of tobacco virus 1 in various tobacco plant tissues placed in 2 moist soils and exposed to winter conditions in Wisconsin*[Wisconsin (S) soil (pH 8.2) and Wisconsin (L) soil (pH 5.2) used,<sup>1</sup> experiment begun Dec. 4, 1934]

Date	Minimum temperature over preceding period	Average number of lesions <sup>2</sup> on 3 leaves of hybrid inoculated from—						Total number of lesions
		Roots	Stubble wood	Stalks	Sucker stems	Leaf lamina	Green plants	
Dec. 4, 1934	°F.	190	37	38	270	1,200	900	2,635
Mar. 5, 1935	—22	136	88	66	37	553	368	1,248
Apr. 3, ———	13	47	38	50	23	475	380	1,013
May 2, ———	21	27	17	15	5	177	147	388
June 2, ———	30	36	52	3	11	200	363	665
June 20, ———	43	12	18	5	18	175	462	690

<sup>1</sup> See explanation of letters on p. 272.<sup>2</sup> Average of readings from 2 different soils.

In a second experiment, the various plant parts were added separately to two different moist soils (Wisconsin (S) and Wisconsin (L) soils) in 6-kg lots placed in open wooden boxes set outdoors in early autumn in a shelter and covered with heavy canvas; hence, they were exposed to considerable microbial action before winter temperatures set in. The soils were kept moist by regular addition of water. Inoculations were made at intervals from the plant parts and from similar dried tissues kept as stock material (table 14). The volume of roots, stubble wood, stalks, and sucker stems taken as samples for extraction with 10 cc of water could only be roughly estimated, so that the relative value of the lesion counts obtained is less than in trials where measured quantities of soils were taken. Samples from soils infested with dried leaf lamina or with virus extract were taken by the usual cork-borer method.

The results are significant chiefly in showing the period the virus may persist in plant parts exposed to decay under more or less natural over-wintering conditions, and at the same time showing that a gradual reduction of virus content occurs in the tissues. Virus was recovered from the plant parts in the soil as long as 8 months after the beginning of the experiment. At the end of this period, the tissues were in a partly decayed condition, though not so markedly so as in the preceding experiment conducted in the absence of soil. Freezing of the soil did not cause any rapid inactivation of virus present in the plant tissues.

A third experiment, conducted along similar lines with the same soils but started later in the season (Dec. 4), at about the time of the first killing freeze, yielded similar results (table 15). It appears from these tests that the virus present in plant tissues below the soil surface is as resistant to inactivation as the tissue is to decay, i.e., some virus may remain in the tissues in moist soil until they are completely disorganized by decay, provided, of course, that excessive leaching out does not occur in the meantime. This was avoided in the present experiments since the soil lots were protected from direct rainfall. No significant differences were observed in the effect of decay upon the rate of inactivation of the virus in the respective plant parts.

Preliminary tests have shown, however, that when virus-carrying plant tissues in advanced stages of decay are exposed to air-drying, the virus may be rapidly inactivated (table 16), presumably as a result of drying, as in the case of virus extract in desiccated soil. The problem of weathering of infected plant tissues on or above the soil surface may consequently be quite different from that dealing with the persistence of the virus in tissues overwintering beneath the soil surface.

TABLE 16.—*Influence of air-drying on rate of inactivation of tobacco virus 1 in badly decayed plant tissues*

[Tissues exposed to decay for 24 weeks at 2°-3° C. before air-drying]

Tissue	Lesions on 3 leaves of hybrid inoculated from—		Tissue	Lesions on 3 leaves of hybrid inoculated from—	
	Tissues air-dried for 8 days	Tissues not air-dried (control)		Tissues air-dried for 8 days	Tissues not air-dried (control)
	Number	Number		Number	Number
Roots.....	2	76	Cured leaf.....	83	550
Stubble wood.....	0	240	Green leaf.....	11	600
Stalks.....	0	400			

#### SURVIVAL TIME

Evidence has previously been presented (6) that the tobacco mosaic virus may overwinter in the soil, that is, strictly speaking, may survive in the soil from the time of the first killing fall freeze to the time of planting in the spring. This period is naturally longest in the more northerly tobacco-growing districts. The maximum length of this period is usually not more than 8 months and may sometimes be considerably less in the North, while in southern tobacco-growing districts it may average less than 2 months.

A number of experiments have been conducted to test the persistence of the tobacco mosaic virus in different soils under various conditions. Experiments have already been described showing the persistence of the virus for at least 8 months in different plant tissues in the soil when exposed to natural winter conditions in Wisconsin (tables 14, 15). The results presented in table 14 show that the virus is able to survive the overwintering period in small portions of infected tobacco plants and that considerable active virus is still present in the decaying tissues at planting time the following spring. The data suggest that the roots and stubble of the plant are not more conducive to overwintering of the virus than is leaf tissue under the same environmental conditions. In the field, leaf tissue may in some instances be a particularly common form of virus-carrying refuse from both first and second growth. Consequently, it may follow that the actual amount of virus overwintering in plant tissues will depend greatly on certain cultural practices, such as time of plowing, and upon other environmental factors, e.g. precipitation, to which infected plant tissues are exposed. However, it is not proposed to discuss these more practical aspects in detail at present.

Other tests of the persistence of virus in the soil have been made, using inoculum in the form of extract, which permitted a fairly uniform distribution of the virus throughout the soil.

In one experiment, virus was added as extract in the usual manner to 2-kg-lots of moist soil in covered pans. Ten different soils were used, as shown in table 17. The pans were placed in a shelter outdoors in the early fall (Sept. 26) for exposure to natural winter conditions, and samples for inoculation were taken at intervals until the middle of the following February. The results presented in table 17 are indicative of the general trend of subsequent trials in that no significant differences were apparent between most of the soils tested, though a few seemed to be definitely less favorable to the survival of the virus in the soil. The virus content of most soils remained at a high level for about 2 months and then dropped suddenly in all soils during December. This is believed to be a result of freezing of the soil (tables 10, 11, and 12), since the sudden decline was correlated with a marked drop in outdoor temperatures below freezing, as indicated in table 17. The experiment was then repeated with soils stored at room temperatures and not exposed to either freezing or drying. Ten of the more outstanding soils were selected on the basis of their behavior in previous trials, and these are listed in table 18. This table shows that inactivation proceeded considerably more slowly in the West Virginia and New York soils, for example, than in the Wisconsin (C) and Pennsylvania soils, but that it was slow in most soils as compared with pure sand. Similar results have been reported in earlier comparisons of sand and soil (6), but the theory previously offered that this phenomenon is related to aeration seems no longer tenable.

TABLE 17.—Persistence of tobacco virus 1 added in the form of extract to different moist soils exposed to winter conditions in Wisconsin

Date	Minimum temperature over preceding period	Lesions on 3 leaves of hybrid inoculated from soil <sup>1</sup> from—									
		South Carolina (2)	Wisconsin (B)	Maryland	Wisconsin (E)	Connecticut	Wisconsin (W)	Tennessee (C)	West Virginia	Wisconsin (C)	Pennsylvania
	°F.	Number	Number	Number	Number	Number	Number	Number	Number	Number	Number
Sept. 26.....		347	230	322	146	259	176	535	200	294	251
Oct. 3.....	37	537	827	875	530	610	354	239	426	593	254
Oct. 17.....	44	900	850	1,000	720	441	588	172	174	284	292
Oct. 21.....	42	600	740	420	674	205	328	165	303	110	312
Nov. 13.....	26	367	357	307	239	503	168	32	157	32	75
Nov. 28.....	26	720	378	329	366	201	204	318	158	105	80
Dec. 18.....	4	62	4	35	12	9	15	0	14	2	1
1935											
Jan. 16.....	-8	1	0	1	2	1	2	1	0	0	0
Feb. 15.....	-22	0	0	0	1	0	2	5	0	0	0
Average.....		393	376	365	299	248	204	163	159	158	141

<sup>1</sup> See explanation of letters and numerals on p. 272.

TABLE 18.—Persistence of tobacco virus 1 added in the form of extract to different soils stored at moderate temperatures and maintained in a moist condition

Soil <sup>1</sup>	Lesions <sup>2</sup> on 3 leaves of hybrid after—						Average number of lesions
	0 week <sup>3</sup>	1 week	5 weeks	9 weeks	13 weeks	16 weeks	
	Number	Number	Number	Number	Number	Number	
West Virginia.....	328	81	350	178	155	322	236
New York.....	127	124	333	240	329	112	211
Ohio.....	583	194	223	17	15	70	184
Wisconsin (B).....	191	169	196	94	180	123	159
Tennessee (S).....	298	175	104	77	29	154	139
Connecticut.....	160	64	265	44	136	143	135
Pennsylvania.....	475	151	28	3	4	23	114
North Carolina.....	196	169	81	64	31	41	97
Sand.....	324	10	2	1	0	0	56
Wisconsin (C).....	189	65	17	6	2	2	47
Virus extract (at 1 to 1,000 dilution).....	44	31	35	40	65		43

<sup>1</sup> See explanation of numerals on p. 272.<sup>2</sup> Average of 2 separate readings.<sup>3</sup> See footnote 2, table 2

A further comparison of the West Virginia and Wisconsin (C) soils with sand is given in table 19. In this test, duplicate series were run at moderate temperatures, 4-months-old and fresh tobacco mosaic virus extract being used respectively. Again the rate of inactivation was lowest in the West Virginia soil and highest in sand, even when fresh virus extract was used, though in all cases the rate of inactivation was higher for the old extract than for the fresh. Similar tests made at the same time with lots of sand screened to four different particle sizes revealed no difference in the rate of inactivation of the virus in the different lots. The reason for this peculiar effect of sand on the virus is not understood.

Additional evidence of the ability of the tobacco mosaic virus in the form of extract to persist for long periods in the soil in the absence of drying and freezing is presented in table 20. In this experiment, virus extract was added to nine different soils stored at room temperatures and kept in a moist condition. The rate of inactivation was slow in all soils, and virus was still recovered from some soils as long as 12 months after the beginning of the experiment.

It was not unusual to find that the ordinary tobacco mosaic virus had become attenuated in varying degrees in soils in which the concentration of virus had become much reduced, that is, in soils yielding perhaps one to five local lesions upon inoculation to a hybrid plant. The presence of these attenuated forms could be demonstrated by inoculating soil samples into ordinary tobacco, which then developed systemic symptoms of varying degrees of mildness. Presumably, in this case, infection resulted from one or a few isolated virus particles, which had become attenuated in some way in the soil. That such attenuated forms represented strains of *tobacco virus 1* could be shown by inoculation back to the hybrid. It has previously been shown that similar attenuated strains can be obtained also from field soils (6).



TABLE 19.—Comparison of the rate of inactivation of tobacco virus 1 added in the form of fresh and of 4-month-old extract to two moist soils of different type and to sand, and stored at moderate temperatures

[Both old and fresh virus extract used in separate tests]

Soil	Total colloids	Virus extract	Lesions <sup>1</sup> on 3 leaves of hybrid after—								Total lesions
			0 week <sup>2</sup>	1 week	2 weeks	3 weeks	4 weeks	5 weeks	7 weeks	9 weeks	
	Percent		No	No	No.	No.	No	No	No.	No	No
West Virginia.....	48.6	Old.....	625	227	61	31	47	54	35	66	1,146
		Fresh.....	850	449	230	167	170	167	236	261	2,530
Wisconsin (C).....	17.4	Old.....	675	300	50	7	15	4	6	10	1,067
		Fresh.....	900	278	132	34	10	10	36	23	1,423
Sand.....	1.8	Old.....	525	42	6	1	4	0	0	1	579
		Fresh.....	538	157	45	35	69	20	42	7	913

<sup>1</sup> Average of 2 separate readings.<sup>2</sup> Immediately after infestation with the virus.<sup>3</sup> See explanation of letter on p 272.

TABLE 20.—Persistence of tobacco virus 1 added in the form of extract to different soils stored at temperatures varying between 50° and 100° F. and maintained in a moist condition

Month	Lesions on 5 leaves of <i>Nicotiana glutinosa</i> inoculated from soil <sup>1</sup> from—								
	West Virginia	Florida	Georgia	Tennessee (C)	South Carolina (1)	Wisconsin (B)	Connecticut	Maryland	Wisconsin (S)
1934	Number	Number	Number	Number	Number	Number	Number	Number	Number
June.....	175	600	350	125	350	600	275		
July <sup>2</sup> .....	60	45	80	8	175	70	12		
August <sup>2</sup> .....	56	50	15	18	12	70	12		
September <sup>2</sup> .....	30	45	23	2	21	68	14	550	543
October <sup>2</sup> .....			28	28	105	310	92	780	196
November.....	250	150	130	10	37	300	34	593	348
December.....	250	130	300	40	200	230	77	441	177
1935									
January.....	170	83	98	37	11	60	43	398	11
February.....	98	42	19	106	12	12	25	96	5
March.....	295	43	15	23	104	33	10	194	35
April.....	120	52	3	16	16	98	8	36	5
May.....	38	30	30	12	30	20	28	152	36
June.....	140	0	0	25	0	76	25	62	162
Average.....	140	103	82	35	80	156	54	330	152

<sup>1</sup> See explanation of letters and numerals on p 272<sup>2</sup> The low figures obtained for these months are due largely to the use of unsatisfactory *Nicotina glutinosa* plants for the tests.<sup>3</sup> Soil accidentally dried out.

These experiments on the survival of the tobacco mosaic virus in the soil under laboratory and greenhouse conditions are believed to give sufficient evidence that the virus may persist regularly in various types of soils and under various environmental conditions for periods of a year or more. It is evident, however, that this survival is accompanied not only by inactivation of by far the greater portion of the virus originally present, but also by attenuation of virulence of a significant part of the remainder. While this high percentage of inactivation is very fortunate in connection with cultivation of the crop, it should be emphasized that after heavily infected crops the overwintering of only a small fraction (1 percent or less) of the virus

originally infesting the soil the previous autumn would yield sufficient inoculum to account for high percentages of infection on the crop the following year.

#### DISCUSSION

The greenhouse and laboratory experiments reported in this paper were not designed primarily in relation to the practical field aspects of the tobacco mosaic problem. It has previously been shown that the tobacco mosaic virus may survive the winter in the soil (6), but little information has been available on its behavior in the soil under the various conditions to which it is naturally exposed. Consequently it was felt that a preliminary study of the individual factors which might play a part in the inactivation or survival of the virus in the soil were fundamental to a further study of the disease under field conditions. The results obtained have yielded an unexpected amount of information in certain respects and should not only aid materially in an interpretation of the apparently inconsistent behavior of the disease in the field, but also in the planning and conduct of further experiments relating to field control.

Apart from field aspects, however, a number of observations have been made during the course of the experiments which may have some bearing on the nature of the virus itself. Such phenomena as the behavior of the virus in sand, its rapid inactivation in soil, in the form of extract, as a result of desiccation and freezing, and its apparent sensitivity to these same conditions in severely decayed tissues suggest starting points for new lines of investigation of a more fundamental nature.

#### SUMMARY

Greenhouse and laboratory experiments were conducted to determine the relative importance of various factors in the persistence or inactivation of the ordinary tobacco mosaic virus (*tobacco virus 1*) in the soil.

The virus was found to leach out readily into the soil from decaying infected plant tissues; consequently, the experiments were performed chiefly with virus extract or different plant tissues added directly to the soil. Representative soils obtained from the different tobacco districts of the United States were used in the investigation.

Addition of virus extract to certain soils resulted in immediate inactivation of an appreciable amount of the virus, but in no case did this approach the high degree of inactivation caused by charcoal or other highly adsorptive substances. Neither did this limited type of inactivation appear to be correlated with the physical character of the soil, in the presence of soil moisture. Desiccation of the soil, however, resulted in an immediate, and usually complete, inactivation of virus extract added thereto. The rate and degree of inactivation during drying was correlated to a considerable degree with the character of the soil. It is possible that inactivation due to drying is related to adsorption phenomena.

The degree of water saturation of the soil above a low minimum, and the range of hydrogen-ion concentration occurring naturally in soils, did not appear to affect the inactivation of the virus. Aeration evidently increased the rate of inactivation both directly and through its influence on microbial activity, but it operated relatively slowly.

Soil temperatures between 5° and 30° C. did not appreciably affect the rate of inactivation of virus extract in the soil. At 40° C., inactivation was definitely greater. Freezing the soil, however, caused rapid inactivation of virus extract in the soil, irrespective of the actual temperature reached, probably as a result of desiccation through freezing out of soil moisture. Neither freezing nor desiccation caused any appreciable inactivation of virus present in undecayed plant tissues in moist soil. Desiccation of severely decayed tissues, on the other hand, caused rapid inactivation of the virus remaining therein.

Exposure of moist infected plant tissues to decay in the presence or absence of soil resulted in a gradual loss of virus content, though considerable amounts of virus remained in the tissues until they became completely disorganized through microbial activity. The rate of this decay was naturally affected by temperature, moisture, and other conditions; nevertheless, considerable amounts of virus remained alive in the tissues after 8 months' exposure out of doors, when kept in soil and consequently not directly exposed to weathering. At temperatures above freezing, virus extract persisted in moist, poorly aerated soil for a year or more. The rate of inactivation was, however, definitely more rapid in some soils than in others, and was strikingly more rapid in pure sand than in field soils, for some reason not at present understood.

It is believed that the results presented should have considerable bearing on studies and observations made on the overwintering of the tobacco mosaic virus under field conditions, as well as on the planning of field experiments on the control of the disease.

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# GROWTH, CHEMICAL COMPOSITION, AND EFFICIENCY OF NORMAL AND MOSAIC POTATO PLANTS IN THE FIELD<sup>1</sup>

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## INTRODUCTION

This paper presents a comparison of the growth, chemical composition, and efficiency of healthy and mosaic potato plants (*Solanum tuberosum* L.) grown in the field under the same conditions. In this investigation the daily efficiency of the lessened leaf spread of mosaic plants is paralleled with that of the much larger leaf spread of normal plants. The problem is: Is a unit leaf area of a mosaic potato plant as efficient per day as a unit leaf area of a normal plant? To determine this point two sets of data are necessary: (1) The daily size and increase in spread of foliage, and (2) the composition in green and dry weight, ash, carbon, starch, and sugar at the end of the measurements.

## WORK OF OTHER INVESTIGATORS

The daily leaf spread had to be determined while the potato leaflets were attached to the plant. These measurements must be as accurate as possible if the results in yield, determined chemically, are to have any value or meaning. Darrow (3)<sup>2</sup> measured strawberry leaves by three methods—leaf product, leaf matching, and adjustable platform planimeter. Correlation between extent of leaf surface and length times breadth of all three leaflets was accompanied by only 0.2 percent error. Less accurate was the matching of leaves to patterns of known areas. The third method, which consisted of inserting the leaf under cellophane and measuring with a planimeter, could be used successfully on attached leaves, as could the first method. From the standpoint of ease of manipulation and accuracy, the leaf-product method appears to be the most successful. Gregory (13) took three linear and two angular measurements on *Cucumis* leaves and from these determined the area as for a hexagon. James (14), working with large numbers of plants, estimated areas by multiplying the average number of leaves by the average area of a well-chosen sample leaflet. Vyvyan (24), using the attached plants as negatives, made prints of bean leaves and determined the area of the prints. Eisele (10) obtained the area of corn leaves by multiplying the length by the breadth by 75 percent. A somewhat similar method was used in this work. Stone (23), from length and breadth measurements, reconstructed potato leaflets on a large scale. The areas of these diagrams were determined by means of a planimeter and reduced to natural size.

The chemical data on normal and mosaic plants, aside from those on yield at the end of the growth period, are fragmentary. Malhotra

<sup>1</sup> Received for publication Apr. 29, 1935, issued March, 1936

<sup>2</sup> Reference is made by number (italic) to Literature Cited, p. 308

(16) found little difference in the percentage of ash of normal and mosaic potato tops. Freiberg (11) confirmed these results with tobacco. Meyer (17) made the general statement that plants which store starch abundantly contain little soluble reducing and nonreducing carbohydrates in their leaves, while others which store no starch accumulate a high percentage of reducing sugars in their leaves. Dunlap (7, 8, 9) reported that mosaic reduced the percentage of reducing sugars, disaccharides, dextrans, starch, and pentosans. Brewer, Kendrick, and Gardner (2), Grandsire (12), Malhotra (16), and Schertz (22) found carbohydrates reduced in mosaic plants, or at least in the pale parts of diseased leaves. Dufrenoy (6) claimed that mosaic inhibited photosynthesis and reduced the sugar content of the leaves.

The practical importance of mosaic lies in the reduced yield of plants affected by it. Orton (19) found that diseased plants were smaller and the yield was 22 percent less than that of normal plants. Wortley (25) reported losses of 100 percent on 200 Triumph plants. Murphy (18) recorded a 58 percent loss in 682 Green Mountain hills. Rosa and Zuckerman (21) planted tuber units of rugose and mild mosaic plants for comparison with similar units of healthy plants. The rugose mosaic plants yielded 45.1 percent and the mild mosaic plants 57.6 percent as compared with the healthy plants.

#### METHODS

Green Mountain potato plants were started in the greenhouse. When of suitable size they were transplanted outdoors in a plot of heavy, wet clay soil that had been treated with ammonium sulphate and potassium nitrate. The plot was surrounded by a cheesecloth screen, 3 feet high, to exclude flea beetles and other insects. The plants were set 4 feet from the screen and 2 feet from each other in order to avoid shading. Whatever the effect, if any, of the cheesecloth, it was shared equally by all the plants. Any insects surmounting this barrier were removed by hand when the daily measurements were taken.

Preliminary plantings were made from the tubers finally selected as seed, to determine the condition of the seed, but since it is not possible to predict with certainty from one seed piece the condition of the other sprouts from the same tuber, the final plants differed in health from the trial plants. However, one normal strong plant remained in apparently healthy condition throughout the experiment, and four showing different degrees of mosaic were chosen for comparison. Each plant was produced by a one-eyed seed piece and only one tuber shoot was allowed to grow.

No attempt was made to name the mosaic according to accepted nomenclature. Two of the plants were slightly affected with mosaic and two were severely affected. Chemical data on the mosaic plants were combined and are presented in two groups, data on slight mosaic and on severe mosaic plants.

Measurements of the leaflets, made daily at 9 a. m., were begun on July 25, when the plants were 10 cm high, and continued until September 2 and 3. By that time the plants had reached the flowering stage and had completed their period of most active growth. Narrow strips of millimeter coordinate paper were used as measures. Three measurements were taken: Plant height, length and breadth of

each tip leaflet, and length and breadth of one of each pair of side leaflets. Leaflets under 7 mm in breadth and 14 mm in length could not be measured accurately. The potato has a compound leaf composed of tip leaflet and two to four paired side leaflets. Throughout this work the tip leaflet was identified as leaflet *a*, the side leaflets as *b*, *c*, and *d*, from the tip to the bottom of the leaf.

To convert the length and breadth measurements of the leaflets into areas, a table was compiled based on their relation to the area of a rectangle of the same dimensions. As a leaflet ages it changes from a long, slender lance shape to an oval with a broad base. For example, the actual area of a leaflet 9 by 17 mm was found to be about 75 cm<sup>2</sup> or 50 percent of 1.53 cm<sup>2</sup>, the area of a rectangle 9 by 17 mm, while the area of an older leaflet 50 by 80 mm averaged 30 cm<sup>2</sup>, or 75 percent of 40 cm<sup>2</sup>, the rectangle area. A table was made up to include leaflets with all possible combinations of measurements. This table by actual trial was found to be as accurate as the method formerly employed by the author (23) and could be used much more quickly.

On September 2 and 3 the plants were removed from the plot. The green and dry weights of roots, tubers, stems, tip leaflets, measured side leaflets, and unmeasured side leaflets were determined.

In the following presentation of data the plant designated 1 was normal, plants 2 and 3 were slightly affected with mosaic, and plants 4 and 5 were severely affected. Only five plants were used in this experiment, for although the measuring was quickly done when the plants were small and the leaves few, as the leaves became more numerous the time required for making and recording measurements rapidly increased to 2 or 3 hours. At the height of growth, over 1,000 measurements were made daily. It would have been impossible to consider the data comparable had they been taken over a period of more than a few hours, which would necessarily have been the case had a larger number of plants been used.

The official chemical method of Munson and Walker (1) was used in making the sugar determinations. Starch was determined by the use of Taka-diastase, according to the method of Davis and Daish (4), adopted by McGinty (15). Ash and carbohydrate were the result of the ordinary combustion procedure.

## GROWTH OF PLANTS

Figure 1 shows that the normal plant produced fewer leaves than any of the diseased plants, except plant 4, but the larger size of the normal leaves gave a greater actual surface exposed. The final area of the measured leaves of the normal plant was 2,000 cm<sup>2</sup>, and that of the measured leaves of the mosaic plants averaged 920 cm<sup>2</sup>. The average area of the normal leaflets was 17.5 cm<sup>2</sup> and that of the mosaic, 7.3 cm<sup>2</sup>. Decrease in leaf area and leaflet size followed closely the increasing intensity of the disease.

Differences in growth of the plants in height did not appear to be significant (fig. 2) until stated in terms of percentage. The gradation from 650 percent total height increase in the normal plant to 148 percent in the most seriously affected plant was consistent with the observable severity of the disease.

The green weight of measured leaflets decreased with increasing severity of the disease (fig. 3). Green weight per square centimeter of leaf area varied, but the dry weight per square centimeter increased

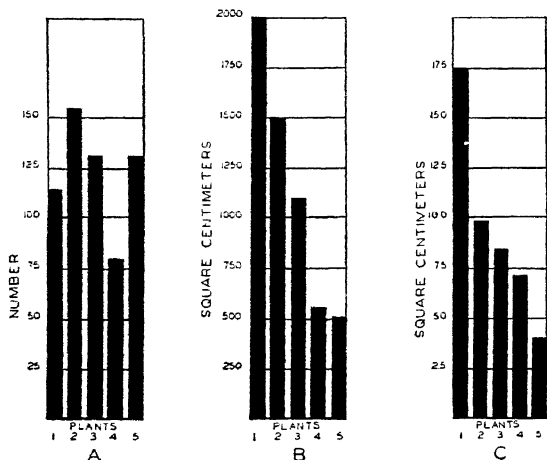


FIGURE 1.—Comparative final number (A), area (B), and average size (C) of all measured leaflets of plant 1, normal; plants 2 and 3, slight mosaic; plants 4 and 5, severe mosaic

with increase in intensity of the disease. Possibly this is an indication of the more compact arrangement of cells in mosaic leaves which Dickson (5) found.

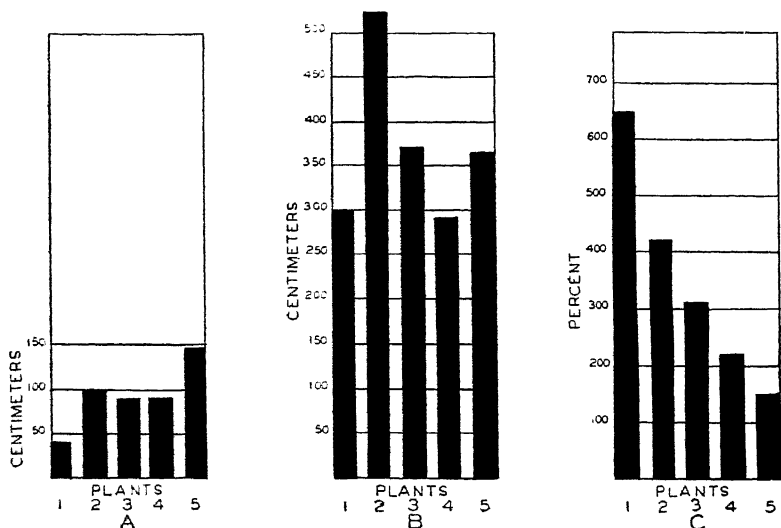


FIGURE 2.—Height at beginning (A) and end (B) of period of measurement, and comparative total percentage increase in height growth (C) of plant 1, normal; plants 2 and 3, slight mosaic; plants 4 and 5, severe mosaic.

Normal leaflets and leaves grew very rapidly for a short time then more slowly. Diseased leaves differed mostly in the amount of growth. They also usually showed an initial spurt, smaller to be sure,

and later they grew more slowly or not at all. Figures 4 and 5 show growth of comparable leaves and leaflets from normal and diseased

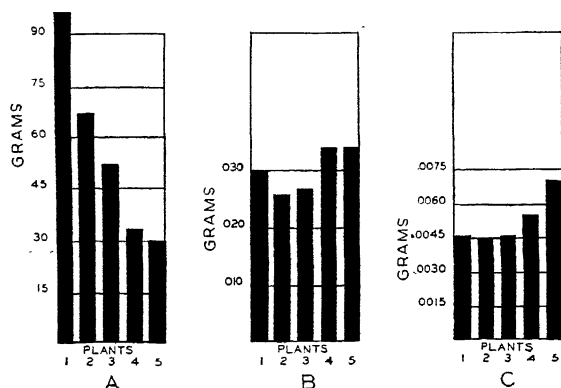


FIGURE 3—Total green weights of all measured leaflets (*A*), and the comparative green (*B*) and dry (*C*) weights per unit of final leaf area of plant 1, normal, plants 2 and 3, slight mosaic; plants 4 and 5, severe mosaic.

plants. Figure 5 indicates that growth in young mosaic leaves was not so far below normal as in older ones, shown in figure 4. The

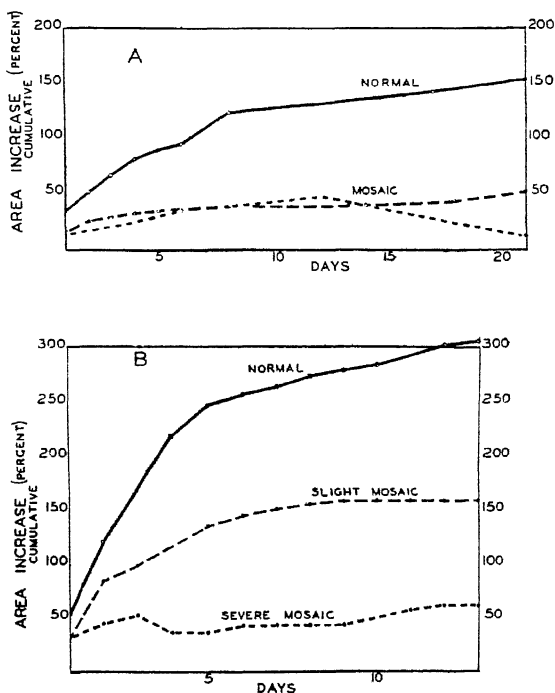


FIGURE 4—Each day's growth of older normal and mosaic leaves and leaflets (percent) added to that of the previous day. Typical comparable normal and diseased leaves (13 in *A*) and tip leaflets (13 *A* in *B*).

growth of leaves was less rapid than that of their tip leaflets because of the much slower growth of the side leaflets. In 87 percent of all



the leaves measured the tip leaflet exceeded the side leaflets in growth, and in 56 percent of the cases leaflet *c* showed the least growth. The tip leaflet is a continuation of the rachis of this pinnately compound leaf. Growth appears to be greatest in the direction of the easiest flow of food material. Pearsall and Hanby (20) thus explained greater tip-lobe growth in ivy.

In apparent opposition to the foregoing is the fact that in the leaflets growth in breadth exceeded growth in length almost without excep-

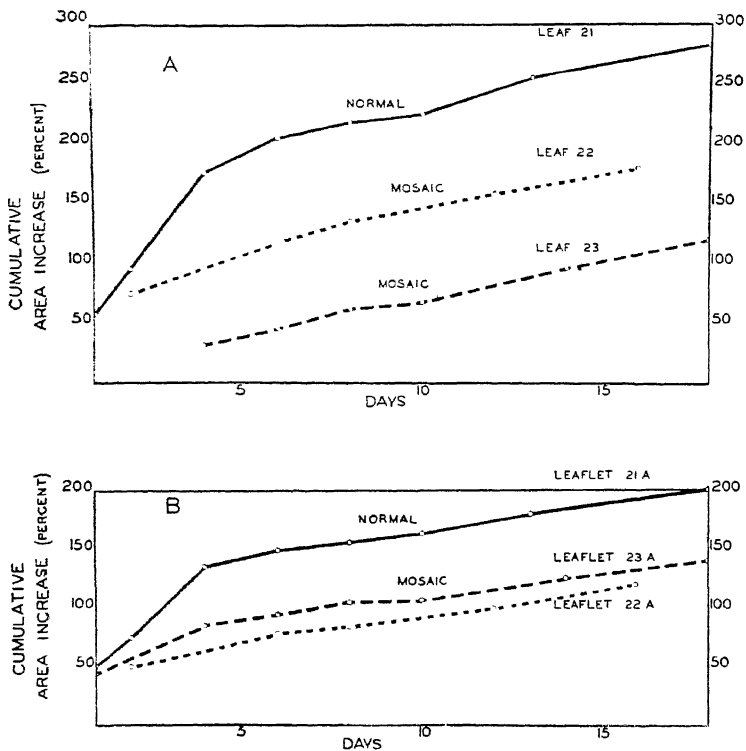


FIGURE 5.—Same as figure 4 but younger leaves in *A* and leaflets in *B*.

tion. The breadth-length growth relationship was more uniform in the normal plant.

In order to show the growth of the whole plant, the growth of every fifth leaf was plotted (figs. 6 and 7). In the normal plant (fig. 6) as growth fell off in one part of the plant it was picked up by a younger part. All normal leaves showed the typical rapid initial growth, but only the earlier mosaic leaves showed an initial spurt. Especially in the severely diseased plant (fig. 7, *B*) growth was without sequence, leaves 40 and 45 being larger and more rapid growers simultaneously with leaves 20 and 25, while leaves 30 and 35 exceeded 40 and 45.

Plant 2 (fig. 7, *A*), slightly affected with mosaic, was intermediate between the severely affected and the normal plants. The initial, accelerated growth was present in nearly all leaves but more subdued.

Growth passed on from leaf to leaf in an orderly manner. In plant 2 rather uniform and small sizes were attained by the leaves. When last measured, leaves of the normal plant were still actively growing and ranged widely in size.

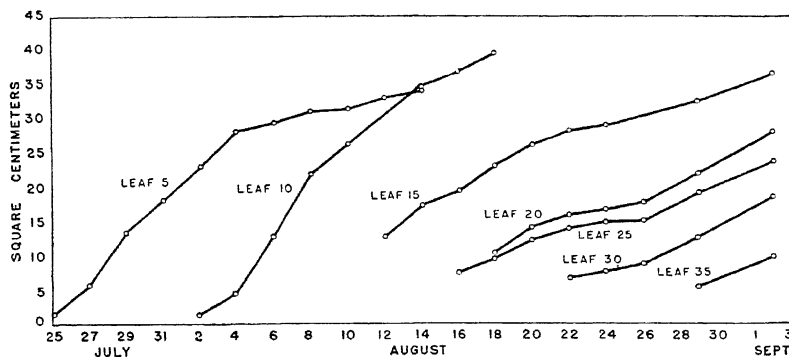


FIGURE 6—Day-by-day area increase (cumulative) of a series of tip leaflets on the normal plant for the entire period of measurement.

Mosaic plants showed decreased daily leaf spread as well as smaller total leaf spread. The relative efficiency of the plants was determined by chemical analysis.

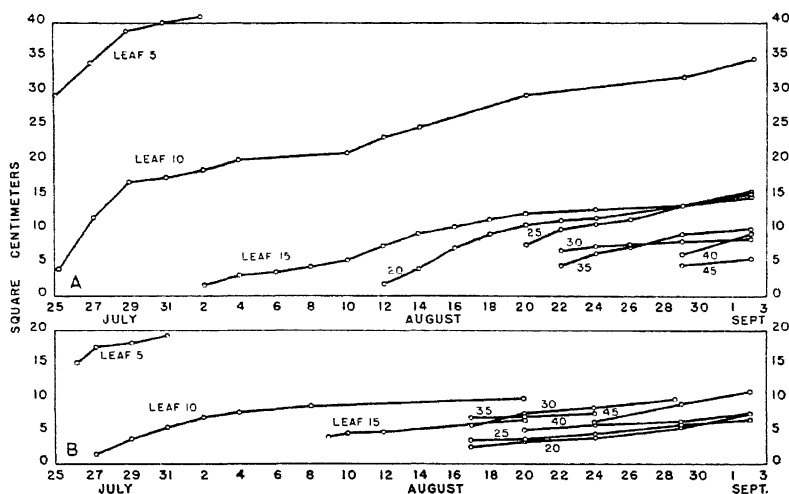


FIGURE 7—Day-by-day area increase (cumulative) of a series of tip leaflets on a slightly mosaic-affected plant, no. 2 (A) and a severely mosaic-affected plant, no. 4 (B).

### CHEMICAL ANALYSIS OF PLANTS

A summary of the results of the chemical analysis is presented in table 1. Data on the two slightly diseased plants (2 and 3) are combined, as are the data on severely diseased plants (4 and 5). The ash content was higher not only in the leaves of diseased plants, as has been reported by other investigators, but also in practically all other

organs and in the entire plant. Carbon varied little among the five plants analyzed. Sugar and starch content were quite generally higher in the normal plant.

TABLE 1.—General results of chemical analyses of normal and mosaic potato plants

Part of plant and condition	Green weight	Dry weight	Ash on dry basis	Carbon on dry basis	Sugar on dry basis	Starch on dry basis
Leaves	<i>Grams</i>	<i>Grams</i>	<i>Percent</i>	<i>Percent</i>	<i>Percent</i>	<i>Percent</i>
Normal.....	96 20	14 33	16 6	38 1	5 46	6 14
Slight mosaic.....	59 64	10 12	16 8	39 1	3 03	4 13
Severe mosaic.....	31 37	5 42	18 7	37 2	3 55	3 71
Stems						
Normal.....	54 93	6 00	18 0	32 8	7 30	3 10
Slight mosaic.....	55 61	6 50	20 7	32 3	2 90	2 40
Severe mosaic.....	23 17	2 70	22 7	31 8	3 30	2 80
Tubers						
Normal.....	172 00	33 25	4 6	38 8	6 10	66 20
Slight mosaic.....	54 85	9 00	6 8	38 2	3 80	53 50
Severe mosaic.....	35 50	6 35	5 0	38 5	6 50	50 60
Roots						
Normal.....	6 50	1 30	21 2	33 3	(1)	(1)
Slight mosaic.....	7 91	1 67	24 2	32 7	(1)	(1)
Severe mosaic.....	3 40	59	12 5	37 8	(1)	(1)
Plant (entire)						
Normal.....	329 60	54 90	9 6	37 8	6 10	43 00
Slight mosaic.....	178 00	27 30	14 8	36 8	3 30	21 10
Severe mosaic.....	93 40	15 10	13 4	36 6	4 70	24 00
Plant (exclusive of roots)						
Normal.....	323 10	53 60				
Slight mosaic.....	170 09	25 63				
Severe mosaic.....	90 00	14 51				

<sup>1</sup> Too small to determine.

Since the ash is made up largely of the elements absorbed by the roots, the relation of ash to root weight indicates the efficiency of the root. According to the data in table 2, the roots of the diseased plants were capable of absorbing about 75 percent as much per unit of weight as were healthy roots.

TABLE 2 —Comparative efficiency of roots of normal and mosaic potato plants

Condition	Dry weight of roots	Ash in plant	Ash per gram of root, dry weight
	<i>Grams</i>	<i>Grams</i>	<i>Grams</i>
Normal.....	1 30	5 26	4 0
Slight mosaic.....	1 67	4 06	2 4
Severe mosaic.....	59	2 01	3 5

The proportion of carbon was fairly constant both among organs of the same plant and in those of normal and diseased plants, though the actual amount of carbon was much lower in the diseased plants.

The distribution of carbon among the organs paralleled more or less the distribution of dry weight (table 3). Mottling had a marked effect on the intake of carbon dioxide, though the degree of mottling appeared to be unimportant. The diseased plants accumulated about 17 percent less carbon than did the normal plant for each 100 cm<sup>2</sup> of leaf surface exposed each day (table 4).

TABLE 3.—*Amount and distribution of carbon in relation to dry weight of normal and mosaic potato plants*

Part of plant and condition	Carbon		Carbon as percentage of total carbon	Dry weight as percentage of total dry weight
	Grams	Percent	Percent	Percent
Leaves				
Normal.....	5.46	38.1	26	26
Slight mosaic.....	3.96	39.1	39	37
Severe mosaic.....	2.02	37.2	36	36
Stems:				
Normal.....	1.97	32.8	10	11
Slight mosaic.....	2.10	32.3	21	24
Severe mosaic.....	.86	31.8	15	18
Tubers:				
Normal.....	12.90	38.8	62	61
Slight mosaic.....	3.44	38.2	34	33
Severe mosaic.....	2.44	38.5	45	42
Roots				
Normal.....	.43	33.3	2	2
Slight mosaic.....	.55	32.7	6	6
Severe mosaic.....	.22	37.8	4	4
Plants (entire):				
Normal.....	20.76	37.8		
Slight mosaic.....	10.05	36.8		
Severe mosaic.....	5.54	36.6		

TABLE 4.—*Carbon in normal and mosaic potato plants in relation to leaf surface exposed per day*

Condition	Growing period	Total carbon	Carbon accumulated per day	Leaf surface exposed over growing period	Surface exposed per day	Carbon accumulated per 100 cm <sup>2</sup> of leaf surface per day
	Days	Grams	Gram	Square centimeters	Square centimeters	Miligrams
Normal.....	40	20.76	0.52	55,561	1,389	37.3
Slight mosaic.....	40.5	10.05	.25	33,307	822	30.4
Severe mosaic.....	37	5.54	.15	17,556	475	31.6

The leaf areas in tables 4 and 7 differ from those in figure 1, for the latter includes the area of only the leaflets actually measured. The presence or absence of the unmeasured twin was carefully noted for later determination of total leaf areas. The method of obtaining the total and daily area exposed over a 3-day period shown in table 5 was applied to the entire period of measurement and thereby the figures in table 4, columns 4 and 5, were obtained.

TABLE 5.—*Data indicating the method of calculating the total and daily leaf area exposed over a 3-day period for normal and mosaic potato plants*

Condition	Leaf area exposed on—			Total area exposed	Average daily area exposed
	Aug. 8	Aug. 9	Aug. 10		
	Square centimeters	Square centimeters	Square centimeters	Square centimeters	Square centimeters
Normal.....	783.64	862.19	920.36	2,566.19	855.40
Slight mosaic.....	435.28	462.03	486.20	1,383.51	461.17
Severe mosaic.....	272.26	288.53	312.61	873.40	291.13

In comparing the daily efficiency of the leaves no account was taken of the fact that the length of day was less during the latter part of the experiment. Since all plants were grown under the same conditions of soil, moisture, temperature, and light, these external factors have not been considered in this investigation. So far as the individual plants were concerned these factors were constant, only the health of the plants varied.

The area of the severe mosaic leaves was actually greater than that recorded since it was impossible to follow the contortions of the wrinkled surface. Hence the severe mosaic leaves were less efficient than they are here pictured.

Diseased plants fell far below the normal plant in the quantity of food manufactured. From table 6 it may be seen that not only the leaves but also the stems and tubers, and indeed the whole plant, when diseased, manufactured and stored less starch and sugar, both in percentage and in weight. Here, again, the severity of the disease had little apparent effect on the proportion of sugar and starch in the plants.

TABLE 6.—*Sugar and starch content and distribution in normal and mosaic potato plants*

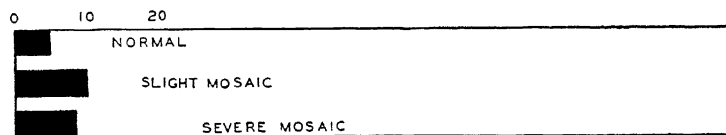
Part of plant and condition	Sugar	Starch	Sugar on dry basis	Starch on dry basis	Sugar as percentage of total sugar	Starch as percentage of total starch
	Grams	Grams	Percent	Percent	Percent	Percent
Leaves						
Normal.....	0.78	0.88	5.46	6.14	24.0	3.8
Slight mosaic.....	.31	.42	3.03	4.13	37.0	7.7
Severe mosaic.....	.19	.20	3.55	3.71	27.5	5.7
Stems						
Normal.....	.44	.19	7.30	3.10	13.5	.8
Slight mosaic.....	.19	.16	2.90	2.40	22.6	2.9
Severe mosaic.....	.09	.08	3.30	2.80	13.0	2.3
Tubers:						
Normal.....	2.03	22.00	6.10	66.20	62.5	95.4
Slight mosaic.....	.34	4.82	3.80	53.50	40.5	89.1
Severe mosaic.....	.41	3.20	6.50	50.60	60.0	92.0
Plant (entire):						
Normal.....	3.25	23.07	6.10	43.00		
Slight mosaic.....	.84	5.40	3.30	21.10		
Severe mosaic.....	.69	3.48	4.80	24.00		

Table 7 bears directly on the efficiency of a unit of leaf surface in relation to the storage of starch. The daily starch production by equal areas of foliage is markedly reduced in the mosaic plants. Since the sugar in the leaves on the final day represented for the most part the result of that day's photosynthesis, sugar content was determined on the basis of the final-day leaf exposure (table 8).

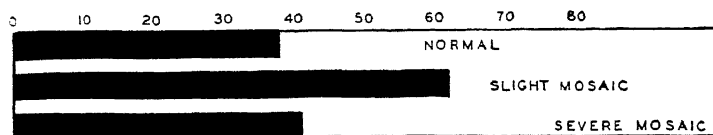
TABLE 7.—*Daily starch production per unit area of leaf exposed in normal and mosaic potato plants*

Condition	Total starch in—			Starch produced per day			Starch produced per 100 cm <sup>2</sup> of leaf per day		
	Tuber	Rest of plant	Entire plant	Tuber	Rest of plant	Entire plant	Tuber	Rest of plant	Entire plant
	Grams	Grams	Grams	Gram	Gram	Gram	Milligrams	Milligrams	Milligrams
Normal.....	22.00	1.07	23.07	0.55	0.03	0.58	39.6	2.1	41.7
Slight mosaic....	4.82	.58	5.40	.13	.02	.14	14.6	2.4	17.0
Severe mosaic....	3.20	.28	3.48	.09	.005	.095	18.9	1.1	20.0

Translocation, as well as actual manufacture, was slower and less complete in diseased plants. Figure 8 represents the percentage of the total starch and sugar content of the plant which remained in the tops at harvest. Mosaic-plant tubers received about 90 percent, whereas the normal tubers contained 95 percent. The diseased-plant tops retained an average of 51 percent of the total sugar of the plant as compared with only 37 percent retained by the normal tops. A



A



B

FIGURE 8.—Percentage of total starch (A) and sugar (B) remaining in the tops of normal and mosaic potato plants at time of harvest

smaller proportion of starch in tubers accompanied a larger proportion of sugar in the tops (table 9). The fact that, in the diseased plants sugar made up about 15 percent of the sugar-starch content, but in the normal plant only 12 percent, further indicated that transfer of sugar and starch was interfered with by the disease.

TABLE 8.—*Sugar content of normal and mosaic potato plants per unit of final-day leaf exposure*

Condition	Total sugar in—			Final-day leaf exposure	Sugar per 100 cm <sup>2</sup> of leaf exposed		
	Leaves	Rest of plant	Entire plant		Leaves	Rest of plant	Entire plant
Normal.....	<i>Gram</i> 0.78	<i>Grams</i> 2.47	<i>Grams</i> 3.25	<i>Sq. cm</i> 3,124	<i>Milligrams</i> 25	<i>Milligrams</i> 79	<i>Milligrams</i> 104
Slight mosaic.....	.31	.53	.84	1,983	16	26	42
Severe mosaic.....	.19	.50	.69	801	24	61	85

TABLE 9.—*Sugar in leaves in relation to starch in tubers of normal and mosaic potato plants*

Condition	Starch in tuber per gram of dry weight of leaves	Sugar in leaves per gram of starch in tubers
Normal.....	<i>Grams</i> 1.54	<i>Gram</i> 0.035
Slight mosaic.....	.47	.064
Severe mosaic.....	.59	.060

The carbon-analysis data of tables 1, 3, and 4 included the carbon of sugar and starch but did not include the small amount of carbon in the ash, about 10 percent. More detailed data on carbon are presented in table 10. The percentage of total carbon which includes the carbon of the ash was even less variable than that of carbon as originally determined. However, the carbon of cellulose, lignin, etc., was quite consistently lower in the normal plant and organs. The normal plant kept more carbon in circulation in the form of sugar and available starch. The normal tubers showed a marked reduction in structural carbon.

TABLE 10.—Carbon, including that in the ash, and carbon exclusive of that in the sugar and starch in normal and mosaic potato plants

Part of plant and condition	Carbon in ash (about 10 percent)	Carbon as computed		Total carbon, 2 and 3		Carbon in sugar and starch (about 37 percent)	Carbon exclusive of that in sugar and starch, 5-7	
		3	4	5	6		8	9
	<i>Gram</i>	<i>Grams</i>	<i>Percent</i>	<i>Grams</i>	<i>Percent</i>	<i>Grams</i>	<i>Grams</i>	<i>Percent</i>
Leaves:								
Normal.....	0.24	5.46	38.1	5.70	39.8	0.61	5.09	35.5
Slight mosaic.....	.17	3.96	39.1	4.13	40.8	.27	3.86	38.1
Severe mosaic.....	.10	2.02	37.2	2.12	39.1	.14	1.98	30.5
Stems:								
Normal.....	.11	1.97	32.8	2.08	34.7	.23	1.85	30.8
Slight mosaic.....	.14	2.10	32.2	2.24	34.5	.13	2.11	32.4
Severe mosaic.....	.06	.86	31.8	.92	34.1	.06	.86	31.8
Tubers:								
Normal.....	15	12.90	38.8	13.05	39.2	8.89	4.16	12.2
Slight mosaic.....	.06	3.44	38.2	3.50	38.9	1.91	1.59	17.7
Severe mosaic.....	.03	2.44	38.5	2.47	38.9	1.34	1.13	17.8
Roots:								
Normal.....	.03	.43	33.3	.46	35.4	0	.46	35.4
Slight mosaic.....	.04	.55	32.7	.59	35.4	0	.59	35.4
Severe mosaic.....	.01	.22	37.8	.23	39.0	0	.23	39.0
Plant (entire):								
Normal.....	.53	20.76	37.8	21.29	38.8	9.73	11.56	21.1
Slight mosaic.....	.41	10.05	36.8	10.46	38.3	2.31	8.15	29.8
Severe mosaic.....	.20	5.54	36.6	5.74	38.0	1.54	4.20	27.8

Yields in the present study must be considered significant in spite of the small number of plants involved. Yields from diseased plants<sup>4</sup> were lower not only because of the decrease in the size of the tops, but also because the diseased tops were only about half as efficient in producing tubers as were the normal tops (table 11).

TABLE 11.—Comparison of tuber weight in normal and mosaic potato plants

Condition	Green weight	Ratio of tubers to tops, in grams
	<i>Percent</i>	
Normal.....	100.0	1.14:1
Slight mosaic.....	68.1	.48:1
Severe mosaic.....	79.4	.65:1

## DISCUSSION

From the data presented it is clear that mosaic exerted a considerable influence on the growth of individual leaves and on the manner in which active growth proceeded. This was apparent both in total growth and in rapidity of growth. Some part of a normal plant was

always growing rapidly, and this region of active growth moved up the stem from leaf to leaf as the new leaves emerged, but in diseased plants this region of rapid growth was less apparent at all times and the rapid area increase could not be followed continuously from leaf to leaf. The mosaic plants produced smaller, and in some cases more numerous leaves, but the total area of leaf surface was consistently less in the diseased plants.

The mere fact, however, that in each case mosaic plants were provided with a smaller working leaf area would have no bearing on the efficiency of the leaves themselves per unit of area. Indeed, smaller leaves might be considered an advantage since they shade each other less and so receive a greater total amount of light per plant. In the mosaic pattern formed by healthy foliage some leaves or parts of leaves are always in the shadow, especially the larger ones towards the base. The advantage of better and more light apparently did not offset the disadvantage of subnormal chloroplasts, for the starch and sugar content in diseased plants was far below that of a normal plant on a leaf-area unit basis. Even in the slightly affected plants in which the mosaic spots were not conspicuous the chloroplasts were incapacitated to a considerable degree and did not function so well in fixing carbon (table 4). The fact that the severely diseased plants seemed to fix more carbon per unit area than the slightly diseased plants may be explained by the inaccuracy of the measurements caused by the wrinkled surface.

Functions in which chloroplasts are not involved seemed to be little affected by the disease. The ash percentage in the diseased plants varied little from that in the normal plant, indicating that roots are nearly normal despite mosaic on the above ground parts. These results seem to locate the detrimental effects of the disease in the leaves.

#### SUMMARY

Daily measurements were made from July 25 to September 3 of all leaflets on a normal potato plant, on plants slightly affected with mosaic, and on plants severely affected with mosaic.

The mosaic plants tended to produce more leaves than the normal plant, but the leaf surface of the former was less than that of the latter, since the smaller number of leaves on the normal plant was more than offset by the greater size of the leaves.

The mosaic plants lengthened more slowly than the normal plant, but gave the impression of greater height because of their spindling growth as contrasted with the stocky growth of the normal plant.

Green weight per unit area of leaves became smaller as the disease affected the plants more severely, but dry weight in mosaic plants was greater per unit area than in the normal plant.

Growth in mosaic leaflets paralleled that in normal leaflets, but always at a slower rate. Mosaic leaflets did not show the marked early rapid growth of normal leaflets, and soon reached the limit of increase in area, so that eventually all the leaves were fairly uniform in size. Normal leaves, on the other hand, continued to grow steadily and maintained a variety of sizes.

In both normal and mosaic plants tip leaflets grew more rapidly than side leaflets, and side leaflets nearest the tip grew more rapidly than those farther back on the leaf petiole. The growth of the



leaflets was always greater in width than in length, and their rate of growth was inversely correlated with their size, the growth rate decreasing as the area of the leaflet increased.

Chemical analysis of the same plants which had been measured for growth showed an ash content lower in normal than in mosaic plants, but this ash content, in proportion to the dry weight of the roots of normal and mosaic plants, showed only a slight reduction in the absorbing ability of the root system of plants affected by disease.

The carbon content in grams seemed to be determined by the size of the plant and varied accordingly, but the carbon percentage of the dry weight varied little in normal and diseased plants.

The special contribution of the entire work, both on growth and analysis, lies in the data which show that mosaic, either slight or severe, reduces the efficiency of a unit area of foliage exposed, as measured by the amount of carbon dioxide fixed in the body of the plant as carbon or stored in the aboveground part of the plant or in the tubers as starch. The point to be emphasized is that the carbon-fixation apparatus does not work so efficiently in mosaic-affected plants as it does in normal plants.

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# ORIGIN AND PRODUCTION OF MORPHOLOGIC AND PATHOGENIC STRAINS OF THE OAT SMUT FUNGI BY MUTATION AND HYBRIDIZATION<sup>1</sup>

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## INTRODUCTION

The phenomenon of physiologic specialization in the oat-smut fungi, *Ustilago avenae* (Pers.) Jens. and *U. levis* (Kell. and Sw.) Magn., has received considerable attention during the last decade. In 1924 Reed (5)<sup>3</sup> gave the first evidence that pathogenically different entities exist within these two species when he described two physiologic races each of *U. avenae* and *U. levis*. Further evidence of specialization in the oat smut fungi was presented by Sampson (10), who designated these specialized entities as biological species.

Differences between forms of the oat smut fungi with regard to characteristics on artificial culture media have been demonstrated by Rodenhiser (9), who described 18 physiologic forms of *Ustilago avenae* and 5 of *U. levis*. In a second report Reed (6) presented further evidence of physiologic races in the oat smuts, and in 1929 (7) he described and classified 11 races of *U. avenae* and 5 of *U. levis*. More recently Reed and Stanton (8) reported the occurrence of a hitherto unknown specialized race of *U. levis* on Fulghum, an extensively grown red oat (*Avena byzantina* C. Koch). It seems apparent, then, that the number of recognized races of oat smut fungi is increasing and that there is considerable economic significance in this fact (8).

In view of the fact that these pathogenic strains<sup>4</sup> of *Ustilago avenae* and *U. levis* exist and that their number apparently is increasing, it would seem worth while to determine the nature of their origin.

Considerable speculation has centered about the role of hybridization and mutation in this connection. In fact, there seemed to be little reason to doubt that new strains would arise as a result of hybridization when it was reported by Hanna and Popp (1) and Holton (2) that crosses between monosporidial lines of *Ustilago avenae* and *U. levis* produced hybrid chlamydospores on the host. On the other hand, a very low degree of viability occurred in the F<sub>1</sub> sporidia from chlamydospores of these species hybrids (2), and it appeared that this would weaken the probability of the origin of new forms by this

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<sup>3</sup> Reference is made by number (italic) to Literature Cited, p. 317.

<sup>4</sup> Physiologically distinct entities within *Ustilago avenae* and *U. levis* have been designated variously as physiologic races, biological species, and physiologic forms. These terms may be interpreted as implying one or more differences in physiologic properties. The term "pathogenic strain" is used in this discussion because only the pathogenic properties have been considered, and it seems desirable to use a term which has a definitely limited denotation.

means. As a matter of fact, after further studies, the writer (3, p. 32) stated that—

in view of the high percentage of nonculturability of the hybrid sporidia the probability that this source of new physiologic forms of *Ustilago avenae* and *U. levis* will be a significant factor in the epiphytology of oat smuts seems very remote.

This statement was based on the results of studies in which crosses between monosporidial lines from chlamydospores of species hybrids were used for inoculation studies.

In view of the fact that only about 1 percent of the  $F_1$  sporidia of the species hybrids would grow in culture (2, 3), the tests were necessarily rather limited in scope. The results of more recent studies, however, in which the  $F_1$  chlamydospores were used for inoculum instead of the crosses between  $F_1$  monosporidial lines, indicate rather conclusively that hybridization between species plays an important role in the development of new pathogenic strains of the oat smut fungi. Furthermore, by using  $F_1$  chlamydospores for inoculum instead of crosses between  $F_1$  monosporidial lines, a more comprehensive understanding of the origin of the buff smut of oats (2, 3) and its relationship to *Ustilago avenae* and *U. levis* has been brought about.

#### MATERIAL AND METHODS

A Gothland strain of *Ustilago avenae*, Reed's physiologic race I (?), a Monarch strain of *U. levis*, Reed's physiologic race I (?), and a pure-line strain of the buff smut fungus were used in these investigations. Specimens of Reed's (?) physiologic races were supplied by the late J. A. Faris, who had obtained them from Dr. Reed. The varieties Gothland (C. I.<sup>5</sup> 1898) and Monarch (C. I. 1896) were used as differential host testers, and Anthony (C. I. 2143) was used for the susceptible check to determine the viability of the inoculum under the conditions of the various tests.

Four monosporidial lines were isolated from a germinating chlamydospore of each of the three strains to be tested (3). Intraspecific and interspecific crosses were obtained by inoculating Anthony oats with sexually compatible combinations of these monosporidial lines (3). Inasmuch as the  $F_1$  sporidia are so weakly viable, the  $F_1$  chlamydospores produced by the specific crosses were used for the pathogenicity tests. The three varieties Anthony, Gothland, and Monarch were inoculated with the  $F_1$  chlamydospores, and selections were made of *Ustilago levis* and the buff smut fungus in the  $F_2$  on Gothland and of *U. avenae* in the  $F_2$  on Monarch. The pathogenic reactions of these selections and the pure-line parental strains were compared on the host testers.

It should be stated here that the factors for echinulate spore walls and brownness of chlamydospores of oat smut fungi are dominant over factors for smooth spore walls and hyalineness (3, 4). Hence in *Ustilago avenae*  $\times$  *U. levis* hybrids, smooth spore segregates are homozygous for that character. Also in *U. avenae*  $\times$  buff smut hybrids, smooth hyaline chlamydospore segregates supposedly are homozygous for these characters. Therefore, *U. levis* and buff smut segregates from *U. avenae*  $\times$  *U. levis* and *U. avenae*  $\times$  buff smut

<sup>5</sup> C. I. denotes accession number of the Division of Cereal Crops and Diseases, formerly Office of Cereal Investigations.

hybrids, respectively, are morphologically pure lines and may be obtained as early as in the  $F_2$  generation.

Since the factors for echinulation and brownness are dominant, the  $F_2$  *Ustilago avenae* segregates may be either homozygous or heterozygous, and consequently three or more generations will be required for selection of a morphologically homozygous strain. Thus, in view of the writer's knowledge of the nature of the inheritance of chlamydospore characteristics, it is relatively simple to obtain morphologically homozygous strains of *U. avenae*, *U. levis*, and the buff smut fungus from hybrids between these organisms. On the other hand, very little is known regarding the nature of the inheritance of factors for pathogenicity in the oat smut fungi and it cannot be assumed that a morphologically homozygous strain selected from a hybrid between two pathogenically different strains is pathogenically homozygous. However, by determining the pathogenicity of such a strain in the  $F_3$  generation some indication as to its pathogenic potentialities can be gained. This method was used in these studies, and any  $F_3$  segregate with smooth spores that was obviously different from the parent lines in its pathogenic reaction was considered as a new pathogenic strain, even though it is recognized that there may be further segregation for pathogenicity in such a strain.

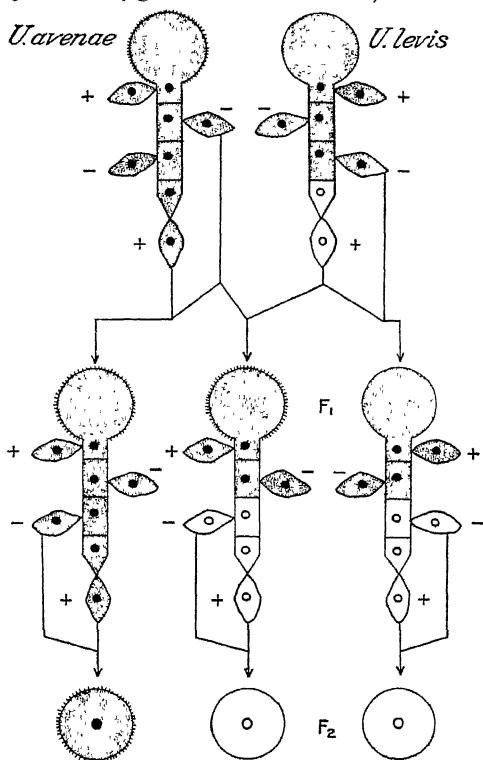


FIGURE 1.—Diagram indicating origin and subsequent appearance of the buff smut fungus which has hyaline chlamydospores. The shading indicates the cells in which the factor or factors for color are carried, and the absence of shading indicates those from which such factor or factors are absent. The nuclei are indicated by dots and circles. The plus and minus signs indicate sporidia of opposite sex.

## RESULTS

### MUTATION

In a previous paper on hybridization between *Ustilago avenae* and *U. levis* (2) a new type of oat smut was described as resulting from a cross between two monosporidial lines which were derived from a species-hybrid chlamydospore. This new smut, which is caused by a fungus that has smooth hyaline chlamydospores, has been designated as the "buff smut of oats" because of the characteristic buff color of the diseased panicles. Inasmuch as it had appeared first in the  $F_2$  generation of a *U. avenae*  $\times$  *U. levis* hybrid, it was suggested in a

later report (3) that perhaps the buff smut developed as a result of the loss of a factor or factors for color or that possibly it was the result of a difference in the factor or factors for color in *U. avenae* and *U. levis*. There are indications, however, that the buff smut fungus has a closer genetic relationship to *U. levis* than to *U. avenae*. Like *U. levis* it has smooth spores, and it crosses with this species more readily than with *U. avenae*. Even so, until now it had not been proved that the buff smut fungus arose directly from *U. levis* and not as a result of hybridization between *U. avenae* and *U. levis*.

The origin of the buff smut of oats has now been established with reasonable certainty and is indicated in table 1 and illustrated diagrammatically in figure 1. Monosporidial lines were obtained by isolating the four primary sporidia from the promycelium of germinating chlamydospores of *Ustilago avenae* and *U. levis*, respectively. Four selfed lines of each species and eight interspecific hybrids were produced by crossing the eight monosporidial lines in all sexually compatible combinations. The color characteristics of the chlamydospores of these selfed lines and species hybrids in the  $F_1$  and  $F_2$ , are indicated in table 1.

TABLE 1.—Color segregation in the chlamydospores of selfed lines and hybrids of *Ustilago avenae* and *U. levis*, showing the crosses and generation in which the buff smut appeared first

[B=brown, SB=some brown, SH=some hyaline]

Generation	Selfed lines of—								<i>U. avenae</i> -10 × <i>U. levis</i> -11							
	<i>U. avenae</i> -10				<i>U. levis</i> -11											
	1×2 <sup>1</sup>	1×3	2×4	3×4	1×2	1×3	2×4	3×4	1×2	1×3	2×1	2×4	3×1	3×4	4×2	4×3
$F_1$ .....	B	B	B	B	$\begin{Bmatrix} B \\ SB \end{Bmatrix}$	$\begin{Bmatrix} B \\ SB \end{Bmatrix}$	B	B	B	B	$\begin{Bmatrix} B \\ SB \end{Bmatrix}$	B	$\begin{Bmatrix} B \\ SB \end{Bmatrix}$	B	B	B
$F_2$ .....	B	B	B	B	$\begin{Bmatrix} SB \\ SH \end{Bmatrix}$	$\begin{Bmatrix} SB \\ SH \end{Bmatrix}$	B	B	B	B	$\begin{Bmatrix} SB \\ SH \end{Bmatrix}$	B	$\begin{Bmatrix} SB \\ SH \end{Bmatrix}$	B	B	B

<sup>1</sup> The numbers refer to the position of the sporidia on the promycelium, beginning at the apex.

The four selfed lines of *Ustilago avenae* produced brown chlamydospores in the  $F_1$  and  $F_2$  generations. In the four *U. levis* selfed lines, all the  $F_1$  chlamydospores were brown while in the  $F_2$  generation brown and hyaline chlamydospores appeared in two of the lines. As shown in table 1, sporidium no. 1 was a component of both the selfed lines that produced the hyaline chlamydospores in the  $F_2$  generation. In the 8 species hybrids, the  $F_1$  chlamydospores were brown, and in 6 of these hybrids the  $F_2$  segregates were likewise brown. In the other 2 species hybrids, however, there was segregation for brown and hyaline chlamydospores, and in both cases sporidium no. 1 of the *U. levis* parent was a component of the cross. On the basis of these results a diagram (fig. 1) was prepared to indicate the origin of the hyaline chlamydospores.

In the *Ustilago levis* parent, segregation for sex occurred in the second division of the diploid nucleus, and during this meiotic process the nucleus of the apical cell of the promycelium lost its faculty for producing color. It has already been reported (3, 4), as indicated in table 1, that brown color is dominant over hyalineness. Consequently all the  $F_1$  chlamydospores of crosses with the monosporidial

line that originated from the apical cell of the promycelium of *U. levis* were brown in color. When these  $F_1$  chlamydospores germinated there was independent segregation for sex and color, or lack of color, and sporidia of opposite sex minus factors for brown color were produced, as indicated in figure 1, and in the  $F_2$  generation the buff smut with hyaline chlamydospores appeared. Therefore, it seems rather probable that the factor or factors for color in the buff smut fungus originated through the process of mutation in *U. levis* and that its ultimate appearance was due to independent segregation of factors for sex and color which made possible a recombination of factors for hyalineness.

#### HYBRIDIZATION

The results of tests to compare the pathogenicity of pure lines, hybrids, and hybrid segregates of *Ustilago levis*, *U. avenae*, and the buff smut organisms are summarized in table 2. The *U. levis* strain produced 99 percent smut on Monarch and 0 percent on Gothland, while the *U. avenae* strain produced 0 percent smut on Monarch and 100 percent on Gothland. The buff smut strain produced 55 percent smut on Monarch and 0 percent on Gothland, indicating that it is somewhat less virulent on Monarch than the *U. levis* strain. These figures show rather definitely that the pathogenic properties of the *U. avenae* strain are distinctly different from those of the *U. levis* and buff smut strains, and for this reason these strains were considered as suitable material to use in hybridization studies.

TABLE 2.—Percentages of smutted panicles produced on oat varieties by pure lines, hybrids, and hybrid segregates of *Ustilago levis*, *U. avenae*, and the buff smut fungus<sup>1</sup>

Variety of oats	Percentage of panicles smutted by —					
	<i>U. levis</i>	<i>U. avenae</i>	Buff smut	<i>U. levis</i> × <i>U. avenae</i>		<i>U. avenae</i> × buff smut
				$F_2$	$F_2$ <i>U. levis</i> segregate $F_3$	$F_2$ buff segregate $F_3$
Gothland.....	0.0	100.0	0.0	22.0	92.0	3.0
Monarch.....	99.0	.0	55.0	21.0	45.0	6.0
						100.0
						22.0

<sup>1</sup> The low percentages of smut produced in the  $F_2$  of the *U. levis* × *U. avenae* and *U. avenae* × buff smut hybrids is no doubt explainable on the basis of a low degree of viability of  $F_2$  sporidia and the production of individuals, through segregation and recombination of factors for pathogenicity, that were not pathogenic on these 2 varieties.

As indicated in table 2, the *Ustilago levis* × *U. avenae* hybrid produced 22 and 21 percent smut on Gothland and Monarch, respectively, in the  $F_2$  generation, and on each variety both *U. avenae* and *U. levis* types were found. From the  $F_2$  generation on Gothland a pure strain of *U. levis* was selected that produced, in the  $F_3$  generation, 92 percent smut on the same variety and 45 percent on Monarch. These figures are in contrast to 0 and 99 percent of smut produced on Gothland and Monarch, respectively, by the *U. levis* parent and 100 and 0 percent produced on Gothland and Monarch, respectively, by the *U. avenae* parent. It is evident, therefore, that by crossing *U. avenae*



and *U. levis* a synthetic strain of *U. levis* has been produced which is more widely virulent than either parent, and that its production was brought about by segregation and recombination of factors for pathogenicity present in the parental lines.

Referring again to table 2, it can be seen that in the  $F_2$  generation the *Ustilago avenae*  $\times$  buff smut hybrid produced 3 percent of smut on Gothland and 6 percent on Monarch, segregates of both types appearing. It was notable that some of the Gothland plants were smutted with the buff smut, whereas this variety was immune from the parent strain of the buff smut. This buff smut segregate from Gothland produced, in the succeeding generation, 100 percent of smut on Gothland as compared with 0 percent of smut produced on the same variety by its buff smut parent. On Monarch the new buff smut strain produced 22 percent of smut as compared with 0 and 55 percent produced on the same variety by the *U. avenae* and buff smut parents, respectively. Thus, apparently the new buff smut strain is decidedly more virulent on Gothland but somewhat less virulent on Monarch than the parental buff smut strain. Furthermore, it is equally as virulent on Gothland and significantly more virulent on Monarch than its *U. avenae* parent. It appears, therefore, to have pathogenic properties not possessed by either parent and apparently is a new strain of the buff smut fungus that has arisen through segregation and recombination of factors for pathogenicity in a hybrid between a strain of *U. avenae* and a strain of the buff smut fungus.

#### SUMMARY AND CONCLUSIONS

A new type of oat smut, described and designated in previous reports (2, 3) as "buff smut", has been found to be the result of mutation in *Ustilago levis*. The buff smut fungus has hyaline chlamydospores, but in all other morphological characteristics it resembles *U. levis*. Consequently, the change that occurred apparently involved only the factor that determines color in the chlamydospores. Evidence is presented which indicates rather definitely that the change in the color factor occurred during meiosis in a germinating chlamydospore, with the result that 1 of the 4 haploid nuclei lost the factor for brownness. This nucleus became located in the apical cell of the promycelium, and all crosses with the monosporidial line derived from this cell gave rise to heterozygous brown chlamydospores in the  $F_1$  generation and the hyaline buff smut spores were segregated in the  $F_2$ . Because factors for brown are dominant over factors for hyalineness, all the  $F_1$  chlamydospores were brown, and the appearance of the hyaline chlamydospores in the  $F_2$  is explained on the basis of independent segregation of factors for sex and color of chlamydospores.

A new pathogenic strain of *Ustilago levis* that attacks Gothland and Monarch was produced by crossing a Gothland strain of *U. avenae* with a Monarch strain of *U. levis*. The synthetic strain of *U. levis* was selected in the  $F_2$  generation from Gothland, and it infected both varieties in the  $F_3$ , being almost as virulent on Gothland as the *U. avenae* parent but somewhat less virulent on Monarch than the *U. levis* parent. Also, a new pathogenic strain of the buff smut fungus that attacks Gothland and Monarch was produced by crossing the Gothland strain of *U. avenae* with the Monarch buff smut strain which would not attack Gothland. The synthetic strain of the buff smut

fungus was selected from Gothland in the  $F_2$  generation, and in the  $F_3$  it was equally as virulent on Gothland as the *U. avenae* parent but somewhat less virulent on Monarch than the buff smut parent.

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## SOME STORAGE DISEASES OF GRAPEFRUIT<sup>1</sup>

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### INTRODUCTION

The storage life of grapefruit (*Citrus grandis* (L.) Osbeck) is greatly limited because of the development of diseases that produce blemishes in the peel. The present publication reports a study of certain of these diseases under various storage conditions.

### DESCRIPTION OF DISEASES

#### PITTING

Pitting, or "pox", the most serious of the storage diseases of grapefruit, has received major consideration in all previous reports. As the term "pitting" implies, the outstanding characteristic of the disease is the development of pits or depressions in the rind of the fruit. In the present paper, pitting is divided into two classes, the definite and the mild.

The term "definite" is used to refer to pits that are 0.2 inch or more in diameter and that are depressed to a fairly uniform depth of about one-thirty-second of an inch (figs. 1 and 2, *A*). The oil glands are usually depressed to the level of the rest of the pitted tissue and sometimes show slightly greater depression. They are rarely elevated above the rest of the depressed area. The pits are seldom normal in color. Occasionally they appear bleached or faded but usually they are darker than the healthy peel, ranging in color from yellow ochre to ochraceous tawny or tawny.<sup>2</sup> They tend to darken with age, yet color can hardly be considered a good index of age.

The term "mild" is used to refer to pitting that is less pronounced than that described above. The pits are usually less than 0.2 inch in diameter and may be relatively shallow although they are often as deep as the definite pits (fig. 2, *B*). They may occur individually or in groups. They sometimes form a zone of small pits around the definite pits (fig. 3) and they may enlarge or coalesce to form definite pits (fig. 4, *A*), but they should not be regarded merely as a stage in the development of the larger pits. The definite pits usually make their appearance without passing through the mild stage and the storage conditions that are favorable to one type of pitting are not always favorable to the other.

#### OLEOCELLOSIS

Oleocellosis of grapefruit differs from pitting in that the oil glands stand out in relief above the collapsed tissue and that the affected

<sup>1</sup> Received for publication June 14, 1935; issued April, 1936.

<sup>2</sup> RIDGWAY, R. COLOR STANDARDS AND COLOR NOMENCLATURE. 43 pp., illus. Washington, D. C. 1912.

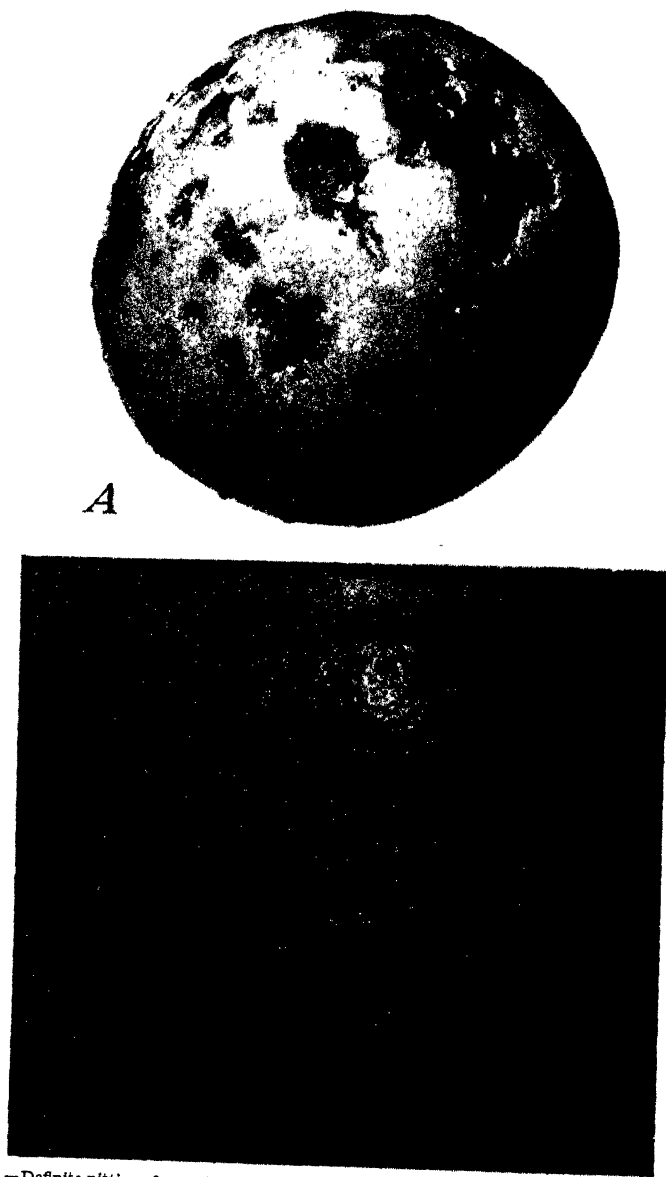


FIGURE 1.—Definite pitting of grapefruit (A) at end of 13 weeks' storage at 36° F. beginning May 5, 1933, and (B) at end of 2 weeks' storage at 34° F. following shipment from Florida to New York.

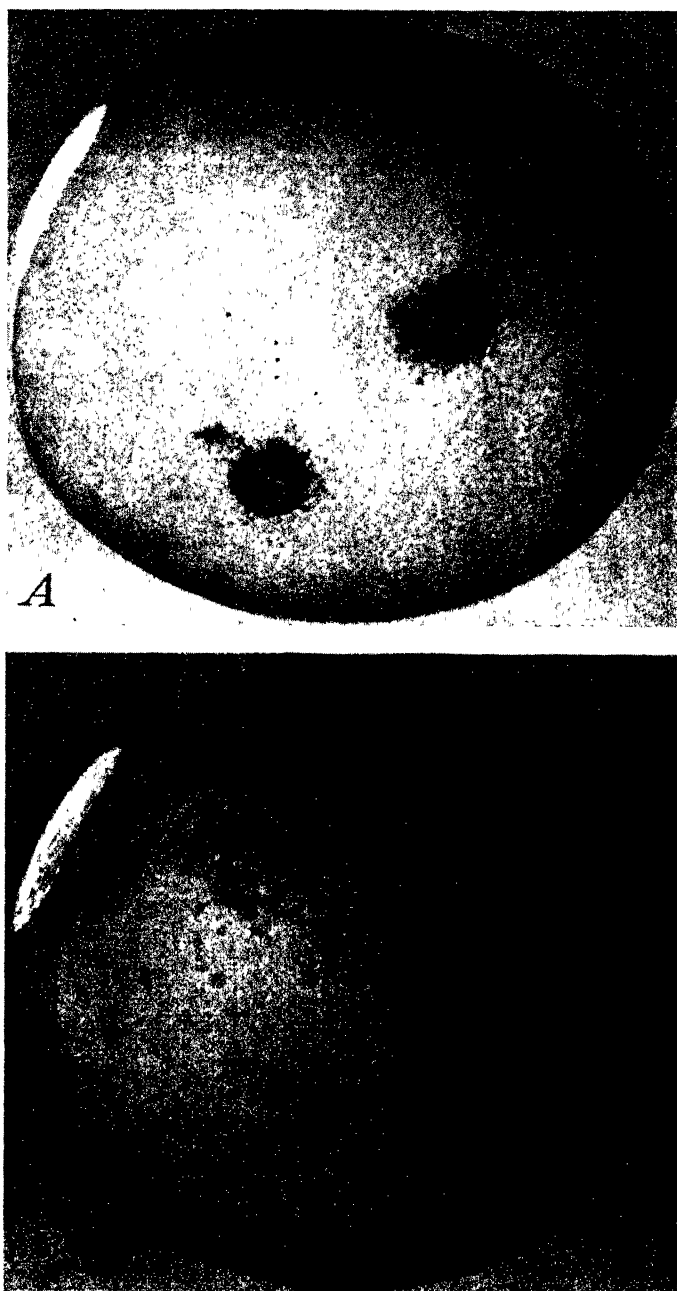


FIGURE 2.—A, Definite pitting of grapefruit at end of 6 weeks' storage at 40° F. beginning February 12, 1934; B, mild pitting of grapefruit at end of 9 weeks' storage, in glycerin wraps, at 32° F. beginning February 12, 1934.

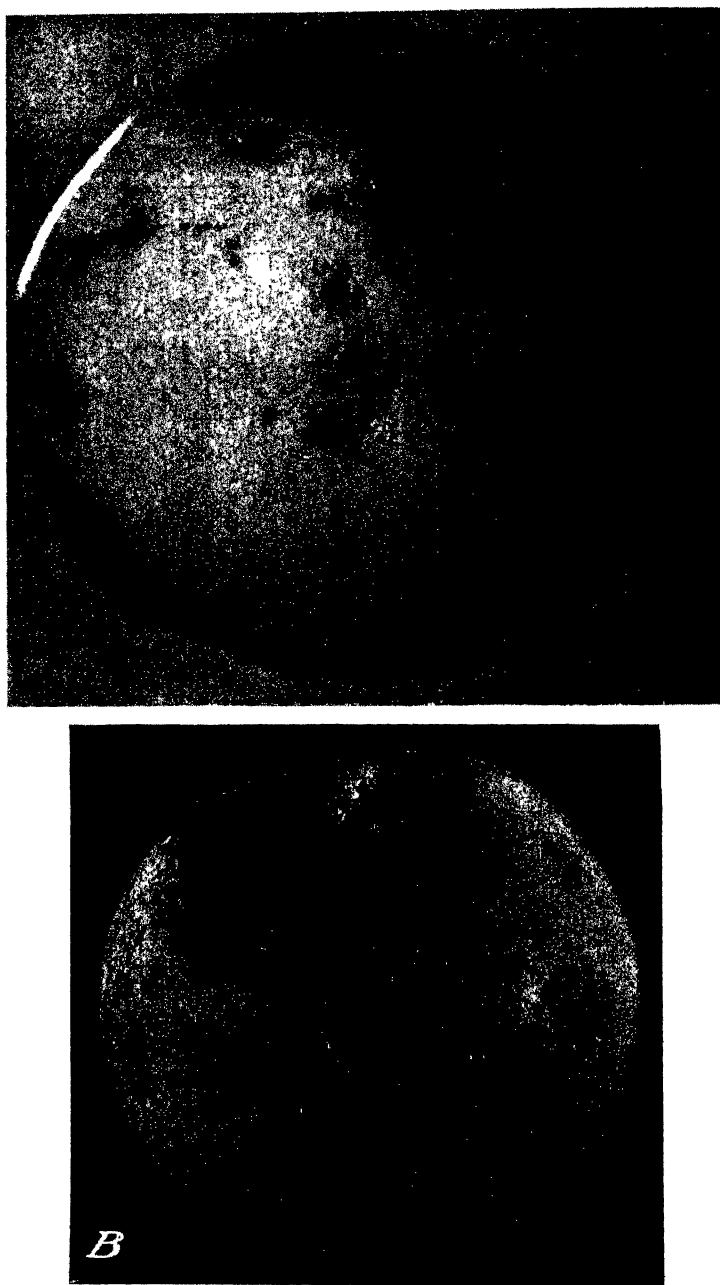


FIGURE 3 —Mild pitting of grapefruit (A), forming a zone around definite pitting at end of 6 weeks' storage at 36° F. beginning March 10, 1934, and (B), forming groups around definite pitting, at end of 9 weeks' storage at 36° F. beginning February 12, 1934.

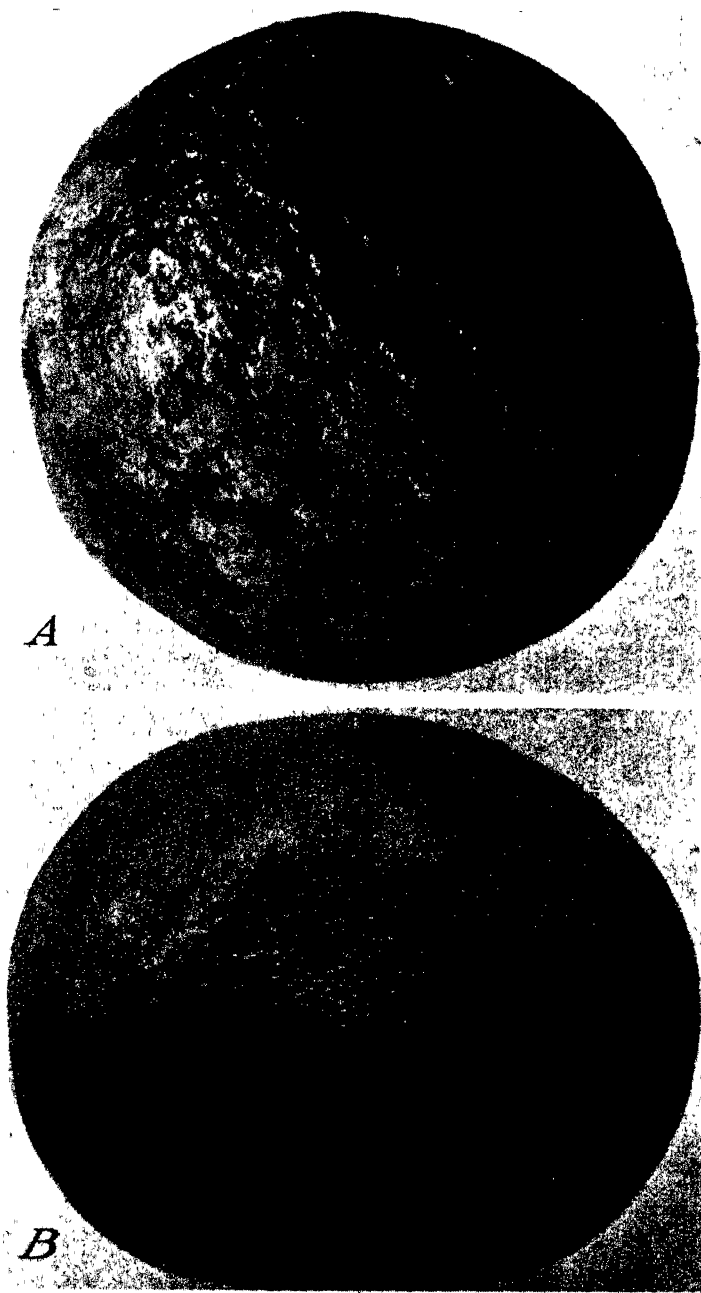


FIGURE 4.—A, Mild pitting of grapefruit, coalescing into definite pitting at end of 11 weeks' storage at 32° F. beginning May 7, 1934; B, oleocellosis of grapefruit, produced by applying expressed juice from grapefruit peel, followed by 4 weeks' storage at 32° F.



areas are usually but slightly depressed (fig. 4, *B*). Its appearance is similar to that of oleocellosis on oranges and lemons (4).<sup>3</sup> As mentioned later, its occurrence is probably initiated by the presence of oil on the surface of the fruit, yet its development may be influenced by storage conditions. Oleocellosis that appears to be due to packing injuries is sometimes referred to as "box scald" or "box rub."

#### SCALD

In addition to pitting, brown stain has been mentioned as a cold-storage disease of grapefruit by Ramsey (12) and scald has been reported by Friend and Bach (5). It would seem probable that the two terms refer to the same disease. In the present publication the term "scald" is used in reference to a superficial and fairly uniform browning covering relatively large areas of the peel (pl. 1, *A*). The disease stands out in marked contrast to all forms of pitting, as the affected areas are not depressed. In mild and typical cases the surface of the peel is firm but in severe cases it may become spongy and soft, suggesting an early stage of watery break-down, which is described later. The color of the scald areas ranges all the way from that of the normal peel to a honey yellow or cinnamon buff. It is seldom as dark as is often found in the case of definite pits.

#### BROWNING OF THE OIL GLANDS

Another type of browning frequently observed is confined entirely to the oil glands. It is referred to in the present report as browning of the oil glands. Many glands in close proximity are affected, giving somewhat the effect of a mass discoloration, yet the tissue surrounding the oil glands usually remains normal in appearance (fig. 5). The color of the spots may range through various shades of brown, as described for scald.

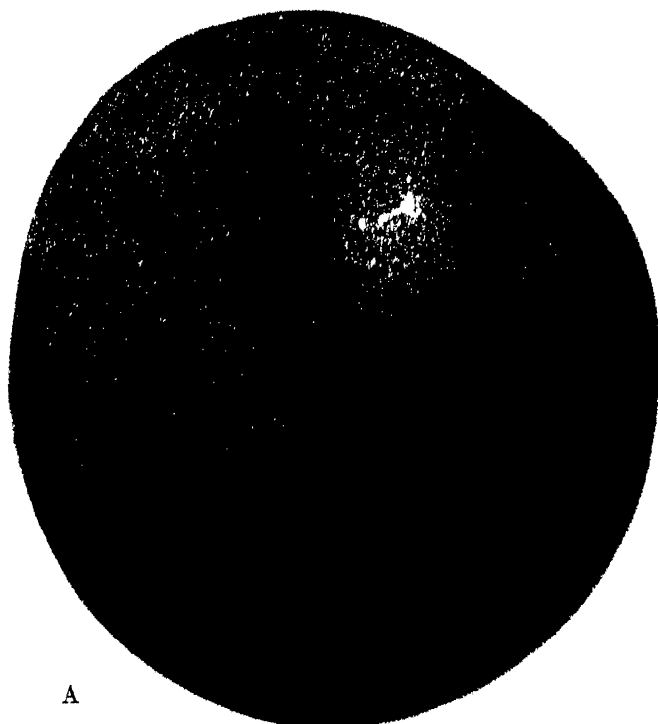
#### WATERY BREAK-DOWN

Watery break-down is a low-temperature disease that has been of rather common occurrence on fruit picked late in the season. The fruit becomes soft, spongy, and water-soaked, giving the appearance of having been frozen (pl. 1, *B*). The diseased condition may be particularly evident either in the peel or in the flesh of the fruit, but more often it is found in both. A study of a cross section of a grapefruit shows a general breaking down of the tissue. The carpels are loosely attached to the inner peel, and when a section of the peel is pressed a watery substance oozes freely from the albedo tissue. Tissue that has collapsed as a result of pits may be swollen so that the once pitted areas are no longer depressed. Affected fruits that are held for a short time at room temperature after removal from storage develop a disagreeable odor of fermentation.

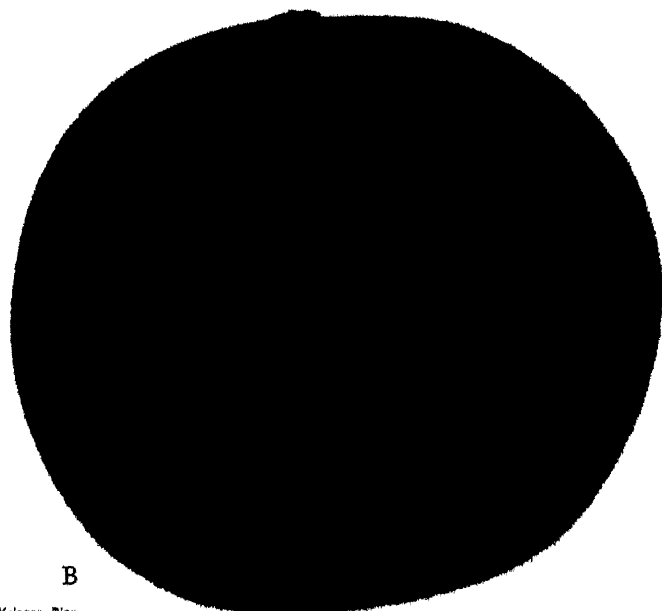
#### ORGANISMS FOUND IN PITTED GRAPEFRUIT

In the spring of 1932 L. F. Butler, then associate pathologist, Division of Fruit and Vegetable Crops and Diseases, United States Department of Agriculture, began a study of the microorganisms found in the pitting lesions of grapefruit. Among a considerable number of organisms isolated, certain bacteria were found which appeared to be pathogenic. When these bacteria were forced into the

<sup>3</sup> Reference is made by number (*italic*) to Literature Cited, p. 351.



A



B

Lith. A. Haen

L. C. C. Krieger, Pinx

A, Scald of heavily waxed grapefruit at end of 10 weeks' storage at 32° F. beginning April 26, 1934;  
B, watery break-down of heavily waxed grapefruit at end of 12 weeks' storage at 32° F. beginning  
February 12, 1934.



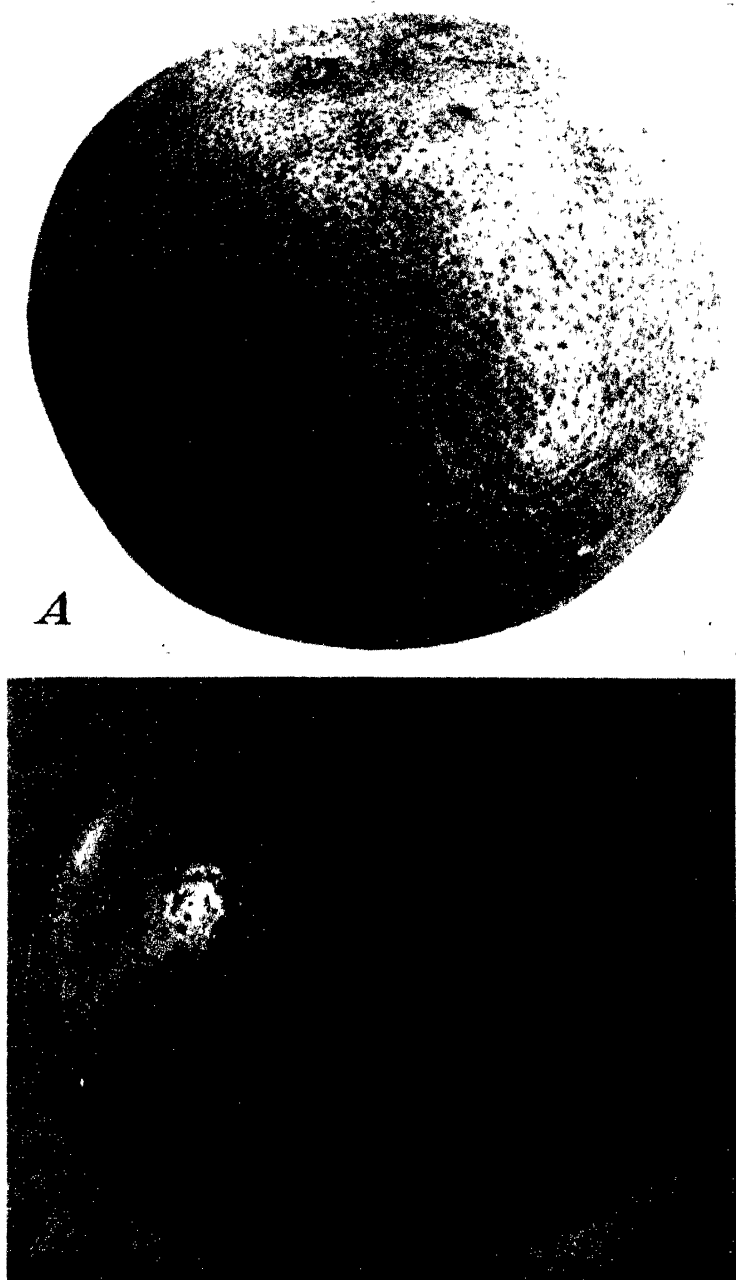


FIGURE 5.—Browning of oil glands on grapefruit (A) at end of 11 weeks' storage at 36° F. beginning May 7 1934, and (B) early stage, at end of 10 weeks' storage at 32° F. beginning Feb. 12, 1934.

peel by means of a hypodermic needle typical pits were produced and the organism could be recovered, whereas injections with sterile solutions failed to produce pits. Following Butler's resignation in June 1932 the further study of the problem was left with the present writers.

Butler found it difficult to obtain any organism from the pitted grapefruit tissue when it was transferred direct to agar, and adopted the method of first placing the tissue in sterile tap water and later transferring drops of the liquid to agar. This method was followed in the isolations reported herein; however, beef broth was often substituted for the sterile water. The usual method of making isolations was to remove the peel and transfer small pieces from the inner surface of the affected tissue direct to the liquid medium. The transfer from the liquid to the solid medium was made as soon as there was evidence of turbidity.

Transfers from normal-appearing albedo tissue (the white spongy portion of the peel) beneath the pits usually remained sterile, whereas transfers that included discolored tissue from the inner surface of the pits gave cultures of bacteria in 67 percent of 360 tests. No single organism predominated, but six or more were isolated repeatedly.

Bacteria were sometimes obtained from the tissue adjacent to the oil glands in normal peel.

The liquid cultures were held for several days after the transfer to solid media had been made, and were then examined for fungus as well as bacterial growth. Mycelium was seldom obtained and when present the fungus was usually found to be a species of *Penicillium*, although cultures of *Colletotrichum*, *Diplodia*, and *Phomopsis* were occasionally obtained (Florida grapefruit).

Microscopic studies were made of the pits in grapefruit that developed without inoculation and of those resulting from inoculations with organisms isolated from pitted tissue. Bacteria were present in large masses in the inoculated material, both in and around the oil glands (fig. 6). In the pits that had developed without inoculation bacteria were occasionally found but never in numbers that would support the conclusion they were the sole cause of the disease.

#### BACTERIA ISOLATED

In the separation and grouping of the various bacteria that have been isolated from pitted tissue considerable attention has been given to their growth characteristics. It will probably be sufficient in this connection to report some of the characteristics of the species or strain that has proved most virulent, originally isolated by Butler, and carried in culture as no. 38.

#### CULTURE No. 38

*Characters*—Motile by 2 to 5 peritrichous flagella;  $0.84\mu$  by  $1.2\mu$  to  $2.5\mu$ ; capsules (72 hours); Gram-negative; colonies on agar smooth, glistening, yellow in center with gray margins; gelatin liquefied, napiform; in nutrient broth clouding medium, odor definite, pellicle enlarging with age and falling to bottom; nitrates reduced to nitrites, no gas; litmus milk slightly acidified, casein precipitated with extrusion of whey.

This description definitely separates the organism from *Bacterium citriputeale* C. O. Smith (13), the cause of black pit of lemons in California, and from *Pseudomonas citri* Hasse (6), the cause of citrus canker, but it leaves some question as to its relation to *Bacillus citri-maculans* Doidge, the cause of a spotting of citrus in South Africa (3).

The present organism is similar to *B. citrimaculans* in most respects but differs from it in the number of flagella and in the reaction to nitrates and to Gram's stain. The writers have not had the opportunity of comparing the two organisms in culture.

The organism (no. 38) has varied in size with growth conditions. It grew well on potato-yeast agar, and on a 24-hour culture of this medium it averaged  $0.84\mu$  by  $2.0\mu$  (fig. 7). When examined in grapefruit 8 days after inoculation it averaged  $0.8\mu$  by  $1.0\mu$ . After 1 or more week's growth in sterilized soil or sterilized tap water its



FIGURE 6.—Bacteria in grapefruit tissues after inoculation with organism no. 38 and storage at  $40^{\circ}$  F. for 13 weeks.  $\times 1,440$ .

size was greatly reduced, but upon transfer to a more favorable medium it regained its former size.

The optimum temperature for the growth of the organism was between  $75^{\circ}$  and  $80^{\circ}$  F., and at temperatures of  $32^{\circ}$  and  $40^{\circ}$  the growth was extremely slow. This temperature response is not in harmony with the temperature conditions under which pitting and other blemishes of grapefruit are most prevalent (p. 324).

The organism has the power of breaking down lemon oil and oil obtained by steam distillation of grapefruit peel. When a layer of either of these oils was floated on top of a beef-broth culture at room

temperature it soon began to darken and finally broke down into hardened brown particles, whereas a similar layer of oil on uninoculated broth retained its normal character and appearance. Experiments were made in which the oil-destroying activity of culture no. 38 was compared with that of 20 other cultures, representing the complete range of bacterial organisms that had been isolated from grapefruit peel. In all cases a correlation was found between the oil-destroying and pit-producing powers. It has been pointed out previously (p. 326)

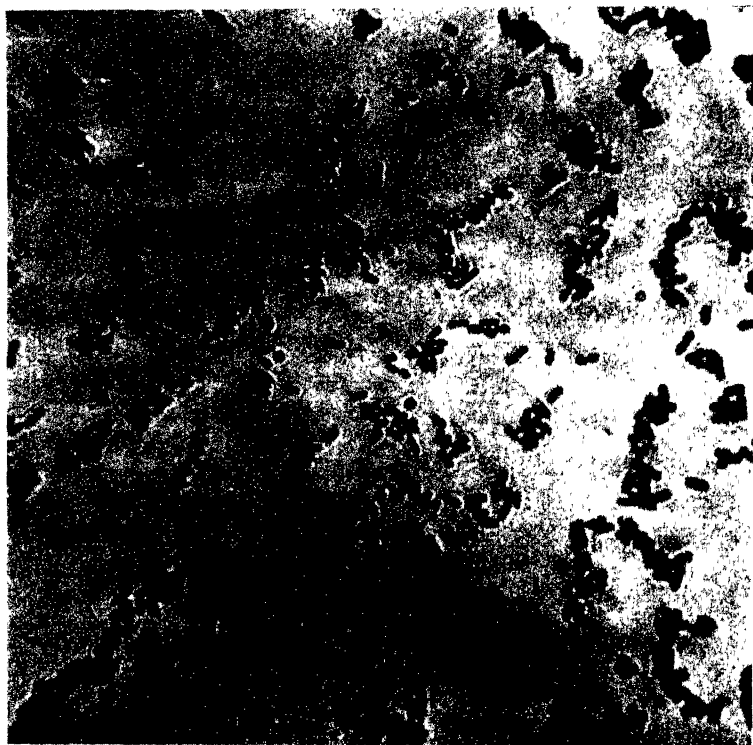


FIGURE 7.—Bacteria (culture no. 38) from a 24-hour culture on potato-yeast agar.  $\times 1,440$ .

that bacteria were found in abundance both in and around the oil glands in pits that had been produced by inoculation.

The presence of bacteria, both in and around the oil glands, and the close correlation between their ability to produce pits and their power of breaking down oil suggests the possibility that the harmful effects of the bacteria may be due to their action upon the oil glands. Bates (1) has recently called attention to the fact that the oil glands of citrus furnish an avenue of infection for decay organisms.

#### INOCULATIONS

Inoculations with pure cultures of the bacteria isolated were made by means of a hypodermic needle. About 0.5 cc of liquid was used in each case. The needle was inserted tangentially and so that the

fluid would be released near the surface of the peel. The inoculum consisted of a heavy suspension of bacteria prepared in sterile water. Control injections with sterile water were made at the same time.

After the fruit had been held for a week to 10 days at 60° or 70° F. or for 5 to 7 weeks at 40°, brown spots 0.5 to 0.8 inch in diameter began to appear at the points where certain bacteria had been released (fig. 8). The spots gradually became darker and more sunken. Isolations made from these spots or pits soon after their appearance usually resulted in pure cultures of the original organism, but when isolations were delayed other bacteria were also obtained.

Bacteria from at least 20 different original isolations gave positive results when injected into the peel, but in spite of considerable variation in virulence and in growth the bacteria from these 20 cultures could probably be grouped under 2 or 3 species. No other organism

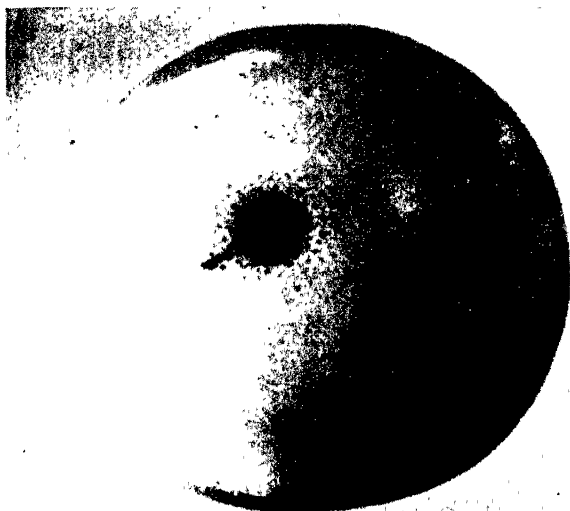


FIGURE 8.—Pit on grapefruit, produced by inoculation with bacteria (culture no. 38), after 7 weeks' storage at 40° F.

has given as strong positive results as that originally isolated by Butler and carried in culture as no. 38.

Injections with sterile water produced no injury when the fruit was held at 70° F., nor at 60°, but in later experiments part of the fruit was held at 40°, and it was found that after 5 to 7 weeks at this temperature definite pitting resulted from the sterile injections in about 50 percent of the tests as compared with 100 percent in similar injections that carried bacterial inoculum. Bacteria were isolated from some of the pits resulting from sterile injections, but in no case did they prove pathogenic when inoculated into the fruit.

Attempts were made to secure invasion by the isolated bacteria without wounding the peel. Grapefruits were immersed in bacterial suspensions and placed under a partial vacuum, with the idea that the return to normal pressure might force some of the bacteria into the peel. Other lots were heated to 100° F. for 1 day or desiccated



for several days and then bathed with bacterial suspensions. None of these treatments caused any increase in pitting.

In experiments reported later (p. 334), in which fruit was obtained direct from the trees, about a tablespoonful of soil from beneath the trees was placed inside each wrapper as the fruit was being packed. After 10 weeks' storage at 32° F. the fruit packed in this manner was found to have several times as much pitting as control fruit packed without the soil. The results led to the use of soil that had been sterilized and then inoculated with the bacteria isolated from grapefruit. The soil was examined before using and was known to have an abundant growth of bacteria. After several weeks in storage the soil-coated fruit had far more pitting than fruit packed in the usual manner. But it was found that when sterile soil was used the increase in pitting was fully as great as with the inoculated soil. The results of these experiments will be discussed further under the heading Humidity and Desiccation (p. 336.)

#### SIGNIFICANCE

The presence of bacteria in a considerable percentage of the pitted tissue and the fact that pits were produced by inoculation with the isolated organisms and the bacteria recovered might be taken as conclusive proof that the disease is of bacterial origin, but there are points in opposition to this simple explanation. Bacteria were found in abundance in pits that were produced by inoculation, but were difficult to locate, if present at all, in pits that developed without inoculation. Although bacteria were obtained in 67 percent of the isolations, less than 10 percent of the cultures were found capable of producing typical pits. At temperatures at which the disease usually occurs pits resulted from sterile as well as from bacterial injections.

Temperature studies showed that the growth rate of the bacteria was extremely slow at 32° to 40° F. and gradually increased up to 75° or 80°, whereas, in studies that follow, it is shown that pitting is worst at 32° to 40° and is almost entirely prevented by holding the fruit at a temperature of 50° or higher.

In view of these facts and others that are developed later showing the sensitiveness of the grapefruit peel to desiccation and to various types of injury, it seems necessary to conclude that while bacteria may sometimes serve as a contributing and modifying factor in the development of pitting, they cannot be considered the fundamental cause of the disease.

The following storage experiments point out some of the factors involved in the development of pitting and other grapefruit diseases.

#### STORAGE EXPERIMENTS

##### MATERIAL AND METHODS

The origin and the packing-house treatment of the fruit used in the various storage experiments are shown in table 1. The different lots will be referred to later by number.

TABLE 1.—Description of the different lots of grapefruit used in experimental storage

Lot no	Size <sup>1</sup>	Date of experiment	Origin	Where purchased <sup>2</sup>	Packing-house treatment	Notes
1A	64	1931 Oct. 27	Orlando, Fla.....	Orlando, Fla.....	None.....	Experiments started after shipment to Washington, D. C., by ordinary express. Do. Do
1B	64	Nov. 27	do.....	do.....	do.....	
1C	64	Dec. 30	do.....	do.....	do.....	
1D	64	1932 Jan. 29	do.....	do.....	do.....	Do. Do Do
1E	64	Feb. 10	do.....	do.....	do.....	
1F	64	Feb. 26	do.....	do.....	do.....	
2	64	Apr. 13	do.....	do.....	do.....	Experiments started before shipment to Washington, D. C., by ordinary express. Do.
3	64	Apr. 16	do.....	do.....	do.....	
4	70	Oct. 19	Cuba.....	Washington, D. C.	Unknown.....	
5	80	Nov. 9	Vero Beach, Fla.....	do.....	do.....	do.
6	80	Nov. 21	Unknown.....	do.....	do.....	
7	80	Dec. 7	do.....	do.....	do.....	
8	80	1933 Jan. 13	Largo, Fla.....	do.....	do.....	Experiments started after shipment to Washington, D. C., by ordinary express. Do.
9A	64	Feb. 11	Brooksville, Fla.....	Brooksville, Fla.....	None.....	
9B	64	do.....	do.....	do.....	Colored and waxed.	
10	80	Mar. 9	Palmetto, Fla.....	Washington, D. C.	Waxed and probably colored.	do.
11	80	Mar. 10	Vero Beach, Fla.....	do.....	Unknown.....	
12	80	Mar. 31	do.....	do.....	do.....	
13	80	Apr. 11	Auburndale, Fla.....	do.....	Waxed and probably colored.	Hauled in bulk by truck from Orlando to Washington, D. C.
14	70	Apr. 14	Orlando, Fla.....	do.....	None.....	
15	64	Apr. 21	Largo, Fla.....	do.....	Unknown.....	
16	70	Apr. 28	Orlando, Fla.....	do.....	None.....	Do.
17	70	May 5	Lake Wales, Fla.....	do.....	Waxed and probably colored.	
18	64	Nov. 16	Brooksville, Fla.....	Brooksville, Fla.....	Colored but not waxed.	
19	64	Nov. 27	do.....	do.....	do.....	Experiment started after shipment to Washington, D. C., by ordinary express. Do. Do.
20	64	Dec. 19	do.....	do.....	do.....	
21	70	1934 Feb. 12	Vero Beach, Fla.....	Vero Beach, Fla.....	do.....	
22	64	Mar. 10	Orlando, Fla.....	Orlando, Fla.....	do.....	Do.
23	64	Apr. 19	do.....	do.....	None.....	Do.
24	64	Apr. 30	do.....	do.....	do.....	Do.
25	70	May 7	do.....	do.....	do.....	Do.

<sup>1</sup> Number of grapefruits per box.<sup>2</sup> The writers are indebted to John R. Winston for cooperation in securing the grapefruit purchased in Florida.

Small lots of fruit were used, but great care was taken to have the different lots uniform. Individual notes were taken on each fruit, and the degree of scald and pitting was recorded on the basis of actual area affected. These individual records were combined and converted into percentages of the total fruit areas. Notes were usually taken at intervals of 2 to 5 weeks, and a number of the lots were held at outside temperature for 1 week after storage before the final note-taking. Unless otherwise stated, the results reported are based on the condition of the fruit while in storage or at the time of removal from storage.

## FACTORS AFFECTING STORAGE DISEASES

## TEMPERATURE

Pitting has been generally recognized as a low-temperature disease, and storage at 45° F. or higher has been recommended as a means of control (4, 5, 8, 9, 12). Friend and Bach (5) found that scald is similarly affected by low temperature. The results of the present studies are in harmony with these conclusions. The development of pitting at different storage temperatures is shown in table 2.

TABLE 2.—Effect of storage temperature<sup>1</sup> on pitting of grapefruit

Lot no.	Fruits under each condition	Storage period	Peel showing—															
			Definite pitting at indicated temperature								Mild pitting at indicated temperature							
			30	32	34	36	40	50	60	30	32	34	36	40	50	60		
			Pct.	Pct.	Pct.	Pct.	Pct.	Pct.	Pct.	Pct.	Pct.	Pct.	Pct.	Pct.	Pct.	Pct.	Pct.	Pct.
4.....	10	7						1.59	0.05					1.10		0.01		
5.....	8	10					.25	0					.46			.01		
9B.....	8	8	0.31			4.00	2.80				1.60		0.34	1.00				
11.....	8	14	1.90			10.70	9.00		0.09		.45		1.40	3.20			0	
13.....	8	13	.07					0	0		0					.01		.02
14.....	8	10	1.30				.74	.50			1.20		0		0			
15.....	9	13	.58			1.40		.01			.09		.34		0			
16.....	8	12	.88			3.60		.28			.40		.17		0			
17.....	9	10	.40			3.80		.06			.13		.30		0			
18.....	24	8	.005				.067	.028			.44			.058		.042		
19.....	16	15	.18			.71		.09			.41		.10		0			
20.....	24	13	.30			.50	1.00	0			.30		.13	.56	0			
21.....	16	8	.04			2.00	1.45	.031			.03		.98	.56	0			
22.....	16	9	.49	0.67	.66	1.50		.51			1.60	1.10	.30	1.90	.26			
23.....	14	8	0.06	.02	.95	10		0		0.08	.01	.19	.01		0			
24.....	24	10	.23	.54	.25			0			.39	.43	.10		.02			
25.....	24	10	0	.06	3.70	3.10	1.90	0		.003	1.80	.65	.72	.24	.003			

<sup>1</sup> All lots with the exception of those at 34° F. were held at a relative humidity of 85 to 90 percent. The 34° lots were held in commercial storage.

After 8 or more weeks in storage there was more definite pitting at 36° F. than at 32° in all of the 12 cases in which these two temperatures were compared, and the average pitting was more than six times as great at the higher temperature (table 2). In a number of the experiments (lots 11, 15, 16, and 17) sufficiently frequent notes were taken to follow the gradual progress of pit development at the two temperatures. The results are shown in figure 9. It will be seen that the above proportion was well maintained throughout the storage period.

In contrast with the foregoing results there was more mild pitting at 32° F. than at 36° in 7 of the 12 tests reported in table 2, and an

average in the 12 tests shows nearly twice as much of this type of pitting at the lower temperature.

When fruit was held at an outside temperature of about 70° F. for 1 week after removal from storage, it was found that there was much greater increase in both forms of pitting on the fruit from 32° storage than on that from 36°. The average for 6 different lots (nos. 19, 21, 22, 23, 24, and 25) showed about twice as much definite pitting on the fruit from 36° storage as on that from 32° and nearly three times as much mild pitting on the fruit from 32° storage as on that from 36°. The final contrast was thus largely in type of pitting with little difference in area of peel affected. The apparently better results at 32° seem to have been due largely to a greater delay in the expression of a condition rather than to a difference in area of peel affected.

The data of table 2 do not justify the conclusion that there was much difference between the pitting at 40° F. and that at 36°, nor between the pitting at 30° and that at 32°. The pitting at 34° was somewhat greater than that at 36°, but the 34° lots were in commercial storage and the condition of the fruit upon removal indicated the probability that the humidity had been lower than with the other lots. Upon removal from storage the increase in pitting on the fruit from 34° and 40° was similar to that on the fruit from 36°, and the increase at 30° similar to that at 32°.

The big contrast, and the significant one that is shown by table 2, is the low percentage of pitting at 50° and 60° F., as compared with that at the lower temperatures. This contrast became still greater after removal from storage. Whereas the percentage of pitting on the fruit from 36° and 40° storage often doubled and that on the fruit

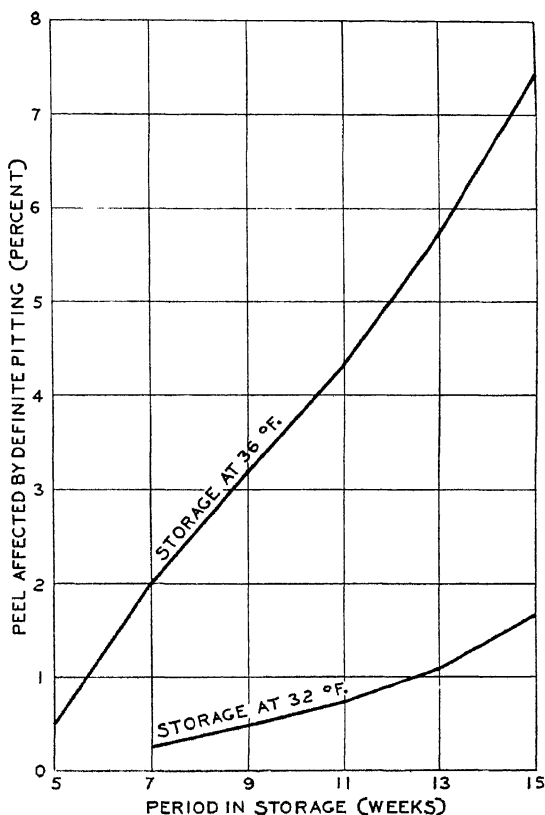


FIGURE 9.—Development of definite pitting on grapefruit held in storage at 32° F., compared with that on grapefruit held in storage at 36°.

from 32° storage often quadrupled within a week, the fruit from 50° and 60° storage showed little or no increase, remaining practically free from all forms of pitting. It has been previously pointed out (p. 329) that injections with sterile water which sometimes caused injury at 40° failed to produce injury at 60° and 70°.

In the later experiments (lots 19 to 25, inclusive) a definite record on oleocellosis was kept. Oleocellosis was of very erratic and rather rare occurrence, and its prevalence apparently was greatly influenced by mechanical injury and packing conditions; but it should be noted that there was occasional occurrence at temperatures of 32° to 40° F., whereas the fruit at 50° remained practically free from the disease.

In the seven experiments with the 1933-34 crop careful records were kept on the browning of the oil glands, scald, and watery break-down. All of these diseases were of erratic occurrence, the susceptibility of the fruit apparently increasing with maturity. All were decidedly more common at 32° than at 36° F. None occurred at 50° (standard packing), and scald and watery break-down were never found at 40°.

Storage temperatures of 50° and 60° F., while preventing pitting and other physiological disorders, favored the development of decay. After 8 weeks at 50° or higher the fruit showed 12 to 60 percent, and averaged about 30 percent, of stem-end rot, whereas the fruit held at 40° or lower usually showed no decay. After longer storage periods there was occasional blue or green mold decay at the lower temperatures. When the fruit was held continuously in storage there was little or no contrast between the decay at 32° and at 36°, but when held for 1 week at room temperature after removal from storage there was about twice as much decay on the fruit from 36° as on that from 32° storage.

After short storage periods the fruit held at 50° or 60° F. seemed to have somewhat the best flavor, but later it became distinctly flat as compared with fruit held at 40° or lower. The fruit held at 36° or 40° seemed in some instances to be superior to that held at 32°.

#### HUMIDITY AND DESICCATION

In a number of the experiments fruit was stored under two different humidities, namely 85 to 90 percent (table 2) and 65 to 75 percent. The comparative results from the two storage conditions are shown in table 3.

In 4 of the 6 tests of storage at 40° F., mild pitting was greater with low humidity than with high humidity and averaged in the 6 tests about 41 percent greater. In all 6 of the same tests definite pitting was greater with low than with high humidity and averaged nearly five times as great. In the 1 test at 50° F. there was not enough pitting with either high or low humidity to be of any great significance. In all of the tests the fruit held at low humidity was more shriveled and had greater loss of weight than that held at high humidity.

It was pointed out on page 330 that placing about a tablespoonful of orchard soil inside each fruit wrapper increased pitting. The results of this and similar treatments are reported in table 4.

The addition of the soil increased definite pitting in 16 and mild pitting in 14 out of a total of 19 tests. An average of the results shows that the soil inclusions caused an increase of approximately 100 percent in both forms of pitting. The increase was as great at 50° F.

as at the lower temperatures. It was somewhat greater with low humidity than with high humidity and fully as great with sterile soil as with inoculated soil.

TABLE 3.—*Effect of humidity on pitting of grapefruit stored at 40° and 50° F.*

Lot no.	Fruits under each condition	Storage period	Storage temperature	Peel showing—			
				Definite pitting		Mild pitting	
				With low humidity <sup>1</sup>	With high humidity <sup>2</sup>	With low humidity <sup>1</sup>	With high humidity <sup>2</sup>
	Number	Weeks	° F.	Percent	Percent	Percent	Percent
4.....	10	7	40	2 19	1 59	1 92	1 10
4.....	10	7	50	04	.05	05	01
5.....	8	10	40	97	.25	.63	.46
9B.....	8	8	40	13 20	2 80	1.40	1 00
10.....	8	4	40	1 10	15	0	.40
11.....	8	4	40	82	.35	0	06
14.....	8	10	40	10 00	74	22	0

<sup>1</sup> Relative humidity 65 to 75 percent

<sup>2</sup> Relative humidity 85 to 90 percent.

TABLE 4.—*Pitting of grapefruit as affected by including soil, sand, or charcoal within the wrapper*

Lot no	Fruits under each condition	Storage temperature	Storage humidity <sup>1</sup>	Storage period	Foreign matter included	Peel showing—			
						Definite pitting		Mild pitting	
						With inclusion	Without inclusion	With inclusion	Without inclusion
	Number	° F.		Weeks		Percent	Percent	Percent	Percent
3	35	32	High.....	6	Grove soil.....	0 95	0 22	0 24	0 007
	10	40	do.....	7	Inoculated soil.....	1 59	1 15	1 10	.27
4	10	40	Low.....	7	do.....	7 36	2 19	2.87	1.02
	10	50	High.....	7	do.....	.55	.05	0	.01
	10	50	Low.....	7	do.....	2.38	.04	1 53	.05
5	8	40	High.....	10	do.....	1 60	.25	1 97	.46
	8	40	Low.....	10	do.....	7 50	.97	12 80	.63
	8	50	High.....	10	do.....	.13	0	0	.01
7	20	40	Low.....	6	do.....	3 80	1 34	4.45	1 84
	20	40	do.....	6	Sterile soil.....	5 47	1 34	5 15	1.84
	6	36	High.....	6	Inoculated soil.....	2 10	1 90	.40	.87
8	6	36	do.....	6	Sterile soil.....	.62	1 90	.65	.87
	6	40	Low.....	6	Inoculated soil.....	2.84	1 00	3.85	1.20
	6	40	do.....	6	Sterile soil.....	2.68	1 00	2.68	1.20
	8	36	High.....	8	Inoculated soil.....	7 20	9 70	1 30	1 70
	8	40	do.....	8	do.....	2 60	5 10	.40	.17
9A	8	40	do.....	8	Sterile soil.....	6 00	5 10	.37	.17
	8	40	Low.....	8	Inoculated soil.....	5 20	3 80	1 10	.74
	8	40	do.....	8	Sterile soil.....	9 00	3 80	3.90	.74
	8	40	do.....	8	Quartz sand.....	9 30	3 80	6 50	.74
13	8	40	do.....	10	Animal charcoal.....	.50	.19	0	0
	8	40	do.....	10	Vegetable charcoal.....	.85	.19	0	0

<sup>1</sup> The low-humidity rooms were held at 65 to 75 percent relative humidity and the high humidity rooms at 85 to 90 percent.

It seemed possible that the increase in pitting might be due to some harmful substance in the soil; quartz sand and vegetable and animal charcoal were therefore tried, but with results similar to those obtained with soil.

The fruit packed with foreign material inside the wrapper usually showed extreme shriveling and loss of weight; this was greater when the foreign material was applied as a moist smear than when held loose in the wrapper. Pitting showed a similar contrast. The shriveling was worse when the fruit was held at low than at high humidity; a similar contrast has been pointed out for pitting, both with and without the presence of foreign material.

In some instances the soil smear was washed off after 5 to 9 days and the fruit was then wrapped in oiled paper, with the result that shriveling and pitting were only very slightly increased by the soil treatment.

In other experiments (lots 21, 22, and 23) glycerin wrappers were used on the fruit in comparison with ordinary wrappers. The results are shown in table 5. The addition of the glycerin increased definite pitting to an average of about 7 times and mild pitting to an average of almost 3 times that with ordinary wrappers. There was an increase in definite pitting at 50° F. as well as at the lower temperatures. There was a relatively high percentage of mild pitting on the treated fruit at both 36° and 32°, whereas definite pitting was much more severe at 36° than at 32°. The fruit packed in glycerin wrappers showed more shriveling and much greater loss in weight than the fruit in untreated wrappers.

TABLE 5.—*Effect of glycerin wrappers<sup>1</sup> on pitting of grapefruit at different storage temperatures*

Lot no.	Fruits under each condition	Storage period	Storage temperature	Peel showing—			
				Definite pitting		Mild pitting	
				With glycerin wrapper	With ordinary wrapper	With glycerin wrapper	With ordinary wrapper
	Number	Weeks	° F.	Percent	Percent	Percent	Percent
21.....	16	8	32	0.32	0.04	3.00	0.03
21.....	16	8	36	12.00	2.00	3.50	.98
22.....	16	9	32	.07	.49	.13	1.59
22.....	16	9	36	7.10	.66	1.50	.30
23.....	14	8	36	2.80	.10	.22	.01
23.....	14	8	50	.32	0	0	0

<sup>1</sup> In lot 22 the wrappers were saturated with glycerin before using, but in lots 21 and 23 glycerin was rubbed on the fruits and dry wrappers then applied. The glycerin was taken up freely by the wrappers.

In the experiments with soil and glycerin the correlation between shriveling and the development of pitting was so close that it seems necessary to consider the drying effects associated with the use of these materials as a factor in the production of the disease. Whether this drying was brought about largely by direct absorption of water or more indirectly by action upon the oil or other peel constituents has not been determined.

There was no increase in the browning of the oil glands, scald, or watery break-down resulting from the use of glycerin wrappers nor from the inclusion of soil within the wrappers.

#### DELAYED STORAGE, HIGH TEMPERATURE, AND TEMPERATURE CHANGES

The sharp decrease in pitting and the accompanying increase in decay at 50° F. as compared with 40° and lower suggested the importance of determining whether similar control of the disease could

be obtained without continued exposure to higher temperatures. With this point in mind three types of experiments were planned: Delayed storage at 50° or higher, exposure to relatively high temperatures before storing, and removal from low-temperature storage at stated intervals.

Hawkins (7) and Hawkins and Barger (8) recommended curing grapefruit before placing it in storage, either by delaying it for a week to 10 days at 70° to 75° F., with a humidity of about 65 percent, or by treating it with gas from a kerosene stove in a coloring room for 3 to 4 days. Both treatments decreased pitting in storage, but the latter method was considered the better because of the lower percentage of decay. Friend and Bach (5) have reported that scald as well as pitting was reduced by holding the fruit at 70° for 10 days before storing.

In the present experiments the period of delayed storage at 60° F. or above was not extended beyond 3 to 5 days, yet it resulted in a definite reduction in pitting. In the four tests reported in table 6 there was an average of about three times as much pitting on fruit that was stored immediately as on that delayed at 60° or above. It should be noted, however, that all lots of fruit used in these tests were purchased on the Washington market and must have been picked several days before the experiment was begun.

In other experiments the fruit was held at 50° F. during the delay. In 3 lots the fruit was held at this temperature for 2 days, in 4 lots for 7 to 10 days, and in 8 lots for 14 or 15 days, before being stored at 32° or 36°. The data in table 6 do not indicate any consistent benefit from these delays. The results from a delay of 7 to 10 days at the higher temperature before storage at 32° and 36° were more favorable than those from a delay of 2 days or 2 weeks.

TABLE 6.—*Effect of delayed storage at 50° F. and above on pitting of grapefruit stored later at 32° and 36°*

Lot no.	Fruits under each condition	Storage period	Storage temperature	Period of delay	Temperature during delay	Peel showing—			
						Definite pitting		Mild pitting	
						With delay	With immediate storage	With delay	With immediate storage
	Number	Weeks	° F.	Days	° F.	Percent	Percent	Percent	Percent
8.....	8	6	36	4	60	0.28	1.60	0.01	1.20
8 <sup>1</sup> .....	8	6	36	4	60	.05	.27	.03	0
11.....	8	7	36	3	70-75	1.25	4.00	.22	.80
15.....	9	13	36	5	70-75	1.10	1.40	.20	.34
18.....	8	12	32	2	50	.13	.53	.25	.25
18.....	8	12	32	7	50	.17	.53	.37	.25
18.....	8	12	32	14	50	.07	.53	.13	.25
19.....	8	15	32	2	50	.35	.18	.32	.41
19.....	8	15	32	10	50	.11	.18	0	.41
19.....	8	15	32	15	50	.77	.18	.06	.41
19.....	8	15	36	2	50	1.70	.71	.08	.10
19.....	8	15	36	15	50	3.70	.71	.07	.10
20.....	16	13	32	7	50	.35	.30	.17	.30
20.....	16	13	32	14	50	.35	.30	.15	.30
20.....	16	13	36	7	50	.22	.50	.40	.13
20.....	16	13	36	14	50	1.85	.50	.10	.13
21.....	16	10	32	14	50	.12	.07	.12	.08
21.....	16	10	36	14	50	1.30	3.30	1.00	2.00
21 <sup>2</sup> .....	16	10	32	14	50	.04	.02	0	.007

<sup>1</sup> In oiled wrappers.

<sup>2</sup> Fruits coated with wax.



In other experiments the prestorage period at the higher temperatures was shortened to less than 24 hours and the fruit was held in an incubator at a temperature usually slightly above 100° F. (table 7). Of the 12 instances in which the fruit was stored at 36° or 40° after exposure to high temperature there were 11 in which definite pitting was decreased, and the average definite pitting for the 12 heated lots was less than one-fifth of that for the fruit stored immediately. The one instance in which definite pitting was not decreased was with fruit heated but 6 hours. Mild pitting was decreased in 6 out of the 8 cases where it was present in the control, and the average mild pitting for the 8 heated lots was about one-third of that for the fruit stored immediately. Of the 11 instances in which the fruit was stored at 32° there was an average of about twice as much definite pitting but less than two-thirds as much mild pitting on the heated fruit as on that which was stored immediately.

TABLE 7.—Effect of prestorage heating at temperatures of 98° F. and above on development of pitting and scald on grapefruit stored at 32° to 40°

Lot no.	Fruits under each condition	Storage period	Storage temperature	Prestorage period of heating	Temperature during heating	Peel showing --					
						Definite pitting		Mild pitting		Scald <sup>1</sup>	
						With heating	Without heating	With heating	Without heating	With heating	Without heating
	Number	Weeks	° F	Hours	° F	Percent	Percent	Percent	Percent	Percent	Percent
9A...	7	8	2 40	20	109	0 26	3 80	0	0 74	0	0
9B...	7	8	2 40	20	109	44	13 20	02	1 40	0	0
13...	8	10	32	6	102	70	04	18	50	37	0
13...	8	10	32	22	105	.09	04	0	50	4 60	0
13...	8	10	2 40	6	102	50	19	0	0	0	0
13...	8	10	2 40	22	105	12	19	0	0	0	0
14...	8	10	32	20	100	3 40	1 30	1 80	1 20	0	0
14...	8	10	2 40	20	100	.03	10 00	08	.22	0	0
15...	9	9	32	17	104	.20	.11	0	.17	7 00	0
15...	9	9	36	6	102	.49	.78	.22	.10	0	0
15...	9	9	36	17	104	.31	.30	0	.10	7 80	0
16...	8	8	32	19	110	.32	2 80	.07	0	6 30	3 50
16...	8	8	36	6	102	.77	2 80	0	0	0	0
16...	8	8	36	19	110	.20	.40	.10	.13	1 20	2 30
17...	9	10	32	6	108	.35	.40	0	.13	12 20	2 30
17...	9	10	36	20	100	.15	3 80	.64	.30	0	0
19...	8	15	32	21	100	.87	.18	.33	.41	0	0
19...	8	15	32	21	100	.20	.18	.10	.41	3	0
19...	8	15	36	21	100	30	.71	.03	.10	0	0
20...	16	13	32	20	98	74	.30	.01	.30	0	0
20...	16	13	32	20	98	03	.30	0	.30	0	0
20...	16	13	36	20	98	.32	.50	05	.13	0	0

<sup>1</sup> Much of the scald bordered upon a watery break-down condition

<sup>2</sup> With low humidity.

<sup>3</sup> Held at 50° F. for 10 days after heating.

<sup>4</sup> Held at 50° F. for 6 days after heating.

The effect of heating and the modifying influence of storage temperature were still more pronounced in the case of scald. Of the fruit that was stored at 32° F. there were 2 lots in which scald was greatly increased by heating and 2 lots in which a high percentage of scald developed on the heated fruit, while the fruit that was stored immediately remained free from the disease. In contrast with this there was only 1 lot in which scald occurred on the heated fruit at 36° and none in which it was found on the fruit that was stored immediately at that temperature. Much of the scald that resulted from heating bordered upon watery break-down. It should be noted that

all of the lots in which scald occurred were picked in April or later. (See table 1.)

In other experiments the fruit was removed from storage to higher temperatures (table 8). At 40°, 50°, and 60° F. the temperature was carefully controlled. The 70° temperature was only approximate, ranging from 65° to 75°.

Of the 15 tests in which fruit was removed from storage at 32° or 36° F. to 70° for 1 day at the end of 1 week and again at the end of 2 weeks (table 8, unwaxed grapefruit), there were 13 cases in which there was less definite pitting and 12 cases in which there was less mild pitting on the fruit that had been moved than on that which remained continuously in storage. The average of the results shows that both forms of pitting were reduced to less than one-fourth of that found on the controls.

TABLE 8—*Effect of temperature changes on storage diseases of unwaxed and of heavily waxed grapefruit*

UNWAXED GRAPEFRUIT

Lot no.	Fruits under each condition		Storage period	Storage temperature	Fruits moved to indicated temperature (° F)	Peel showing—								Fruits showing watery break-down	
						Definite pitting		Mild pitting		Browning of oil glands		Scald			
						With moving	Without moving	With moving	Without moving	With moving	Without moving	With moving	Without moving	With moving	Without moving
No.	Weeks	° F.		Percent	Percent	Percent	Percent	Percent	Percent	Percent	Percent	Percent	Percent		
8	8	6	36	70° for 1 day after 1 week and again after 2 weeks of storage.....	0.57	1.90	0.02	0.87							
9 A	4	8	36	do.....	.51	9.70	0	1.70							
9 B	4	8	36	do.....	.48	4.00	.16	.34							
15	9	14	32	do.....	.20	.58	.01	.09							
15	9	14	36	do.....	.29	1.40	.15	.34							
16	8	8	32	do.....	.97	.30	0	3.50							
16	8	8	36	do.....	.07	2.80	.13	0							
18	8	12	32	do.....	.13	.53	.07	.25							
19	8	12	32	do.....	.12	.46	.50	1.45							
20	8	13	32	do.....	.04	.30	0	.30	0.05	4.50					
20	8	13	36	do.....	.52	.50	.67	.13	.12	.05					
21	16	8	32	do.....	.004	.04	.04	.03	0	0	0	0	0		
21	16	8	36	do.....	.90	2.00	.65	.98	0	0	0	0	0		
22	16	9	32	do.....	.03	.49	.05	1.60	0	0	0	0	0		
22	16	9	36	do.....	.51	.66	.08	.30	0	0	0	0	0		
23	14	8	32	70° for 2 days after 1 week's storage.....	.01	.02	.06	.01	0	0	0	.85	0		
25	16	10	32	do.....	0	.06	.004	1.80	0	0	0	1.30	6.3		
22	16	9	32	70° for 1 day after 2 weeks' storage.....	.04	.49	.12	1.60	0	0	0	0	0		
23	14	8	32	70° for 2 days after 2 weeks' storage.....	.004	.02		.01	0	0	0	.85	0		
25	16	10	32	60° for 2 days after 2 weeks' storage.....	.004	.06	.15	1.80	0	0	21	1.30	12.5		
25	16	10	34	60° for 2 days after 2 weeks' storage.....	.56	3.70	.40	.65	0	0	0	0	0		
25	16	10	36	do.....	.52	3.10	.22	.72	0	0	0	0	18.8		
23	14	8	32	70° for 2 days after 3 weeks' storage.....	0	.02	.08	.01	12	0	.27	.85	0		
25	16	10	32	60° for 3 days after 3 weeks' storage.....	.17	.06	.05	1.80	0	0	0	1.30	0		
25	10	10	32	60° for 3 days after 4 weeks' storage.....	.11	.06	.04	1.80	0	0	0	1.30	0		
18	8	12	32	50° for 1 week after 2 weeks' storage.....	.06	.53	0	.25							
19	8	12	32	do.....	.15	.46	1.17	1.45							
20	8	13	32	do.....	.015	.30	0	.30	.07	4.50	0	0	0		

TABLE 8.—*Effect of temperature changes on storage diseases of unwaxed and of heavily waxed grapefruit—Continued*

Lot no.	Fruits under each condition		Storage period	Storage temperature	Fruits moved to indicated temperature (° F )	Peel showing—								Fruits showing watery break-down	
						Definite pitting		Mild pitting		Browning of oil glands		Scald			
						With moving	Without moving	With moving	Without moving	With moving	Without moving	With moving	Without moving	With moving	Without moving
No	Weeks	° F.			Percent	Percent	Percent	Percent	Percent	Percent	Percent	Percent	Percent	Percent	Percent
20	8	13	36	50° for 1 week after 2 weeks' storage.....	0	.80	0	.50	0	.07	0	.13	0	0	0
21	16	8	32	do.....	.10	.04	.04	.03	.04	0	0	0	0	0	0
21	16	8	36	do.....	.50	2.00	.20	.98	.04	0	0	0	0	0	0
22	16	9	32	do.....	.06	.49	1	30	1.60	0	0	0	0	0	0
22	16	9	36	do.....	.87	.66	.14	.30	0	0	0	0	0	0	0
23	14	8	32	do.....	.16	.02	.05	.01	0	0	0	.85	0	0	0
25	16	10	32	do.....	0	.06	.01	1.80	0	0	.60	1.30	0	6.3	0
25	16	10	34	do.....	.20	3.70	.10	.65	0	0	0	0	0	18.8	0
25	16	10	36	do.....	1	50	3.10	.20	.72	0	0	0	0	18.8	0
24	16	10	32	50° permanently after 1 week's storage.....	.02	.23	0	.39	0	1.10	0	0	0	6.3	0
24	16	10	32	50° permanently after 2 weeks' storage.....	.04	.23	0	.39	0	1.10	0	0	0	6.3	0
25	16	10	32	do.....	.04	.06	0	1.80	0	0	0	1.30	0	6.3	0
25	16	10	34	do.....	.02	3.70	0	.65	0	0	0	0	0	18.8	0
25	16	10	36	do.....	.08	3.10	0	.72	0	0	0	0	0	18.8	0
24	16	10	32	50° permanently after 3 weeks' storage.....	.10	.23	.03	.39	0	1.10	0	0	0	6.3	0

## HEAVILY WAXED GRAPEFRUIT

18	8	12	32	70° for 1 day after 1 week, and again after 2 weeks of storage.....	0	0	0.13	23.20							
19	8	15	32	do.....	0	0.07	.32	2.11	0.32	1.10	0	0	0	0	0
20	8	13	32	do.....	.03	1	.10	.01	.06	.20	9.20	0	0	0	0
20	8	13	36	do.....	.20	.34	.15	.05	0	0	0	0	0	0	0
21	16	10	32	do.....	0	0	.004	.04	0	0	0	0	6.3	25.0	0
22	16	9	32	do.....	.02	.22	.13	.55	0	0	0	0	0	0	0
22	16	9	32	70° for 1 day after 2 weeks' storage.....	.04	.22	.12	.55	0	0	0	0	0	0	0
23	14	8	32	70° for 2 days after 2 weeks' storage.....	.01	0	.03	.02	.50	0	0	12.1	0	4.8	0
21	16	10	32	50° for 1 week after 2 weeks' storage.....	.02	0	.01	.04	0	0	0	0	18.8	25.0	0
22	16	9	32	do.....	.003	.22	.08	.55	0	0	0	0	0	0	0
22	16	9	36	do.....	.20	.18	.01	.20	0	0	0	0	0	0	0
23	14	8	32	do.....	0	0	0	.02	0	0	8.3	12.1	0	4.8	0
24	16	10	32	50° permanently after 2 weeks' storage at 32°.....	.004	.02	0	.03	.18	.22	0	4.3	0	6.3	0

In the few tests in which the fruit was moved to 60° F. instead of 70° or moved to 60° or 70° only once by the end of 2 weeks' storage, the average results were fully as good as those reported above, but in the 3 cases in which the change in temperature was delayed to the end of 3 weeks or later there was little evidence that it caused any reduction in pitting.

In other experiments the grapefruit was removed from 32° or 36° F. at the end of 2 weeks' storage to 50° for 1 week and then returned to the original temperature (table 8, unwaxed grapefruit). The results were not as favorable as with the shorter periods of exposure to higher temperatures, yet out of the 12 tests there were 8 instances in which there was less definite pitting and 10 in which there was less mild pitting on the fruit that had been moved than on that which remained at

a uniform temperature. The average of the results shows that both forms of pitting on the moved fruit were reduced to approximately one-third of that found on the controls.

In other experiments the grapefruit was moved from 32°, 34°, or 36° F. to 50° permanently at the end of 1, 2, or 3 weeks (table 8, unwaxed grapefruit). In the 1 test in which the change was made at the end of 1 week and in the 4 tests in which it was made at the end of 2 weeks, mild pitting was entirely prevented and definite pitting reduced to an average of about 3 percent of that found on the controls. In the 1 instance in which the change was made at the end of 3 weeks, both forms of pitting were far less serious on the fruit that had been moved than on that which had remained continuously at the lower temperature.

With the heavily waxed fruit (table 8) there was usually much less pitting on the controls (at constant temperature), yet the percentage of reduction resulting from short-period exposure at 70° F. or 1 week at 50° was fully as great as with the unwaxed fruit. A similar reduction was obtained in the one test in which the waxed fruit was moved from 32° to 50° permanently at the end of 2 weeks.

In the experiments (table 8) in which browning of the oil glands, scald, or watery break-down occurred, the effect of the various moving treatments was far more pronounced than with pitting. It is interesting to note the number of cases in which there was a high percentage of scald or watery break-down on the controls and entire absence of the disease on the fruit that had been moved.

The data of tables 6 to 8, inclusive, were based on the condition of the fruit while it was still in storage, but the contrasts reported for the various diseases were fully maintained after removal from storage.

The fruit that had been heated was older in appearance and taste and this was occasionally true of fruit that had been delayed or that had been moved for short periods to higher temperatures.

The prestorage heating treatments often resulted in a decided increase in decay and the delays at room temperature sometimes resulted in a slight increase, whereas the various short-period removals to higher temperatures caused no increase in decay. The fruit that was moved to 50° F. permanently at the end of 1, 2, or 3 weeks' storage at the lower temperatures soon developed a very high percentage of decay; that moved at the end of 1 week showed much more decay than that moved at the end of 2 or 3 weeks.

The possible significance of the results from the various temperature changes is discussed later (p. 349).

#### CARBON DIOXIDE

A series of experiments was carried out to determine whether short-period exposure to carbon dioxide gas would have any effect upon the later development of pitting in low-temperature storage. The first tests were made on fruit described as lots 1A to 1F, inclusive (table 1). After shipment from Orlando, Fla., to Washington, D. C., by ordinary express (2 days) the fruit was given 2 days' exposure to atmospheres containing 40 to 45 percent of carbon dioxide gas before being placed in storage at 32° F. Similar lots were held at the same temperatures in normal air. Seven fruits were used under each condition. Most of the tests were made at 77°, 68°, and 49°, but two tests each at 50° and 41° were included. Individual records were kept of each fruit, as described on page 330. After 10 to 12

weeks in storage less definite pitting was found on the fruit receiving the prestorage carbon dioxide treatments in all of 18 tests and less mild pitting in 17 out of 18 tests. In both cases there was an average of one-third as much disease on the treated as on the untreated fruit. The contrasts were greater with the fruit that was held at the higher temperatures during the period of treatment than on that held at the lower temperatures.

In order to keep the percentage of carbon dioxide constant in the above experiments, the carbon dioxide-air mixture was continuously renewed, about five complete changes in the atmosphere being made in 24 hours, whereas the controls were held under moist-chamber conditions with no regulation of the air renewal, thus leaving the possibility of a difference in aeration as well as a difference in carbon dioxide. In later tests the fruit was held in pony refrigerators, with practically the same aeration conditions for the treated and untreated fruit but less accurate control of gas composition and temperature.

The pony-refrigerator experiments were made at Orlando, Fla., in the spring of 1932. Two crates of grapefruit were used under each condition. The refrigerators were cooled by ice, the temperature of the treated fruit dropping to 50° F. in about 18 hours and remaining there till the end of the experiment, and the temperature of the control fruit running 2° to 3° higher. The carbon dioxide was supplied from the usual type of carbon dioxide cylinder. It was run rapidly into the test refrigerator for 30 minutes and the rate was then decreased till it approximately balanced that of the leakage. In an experiment started on April 13 the carbon dioxide was held at about 20 percent and the treatment was continued for 30 hours. In an experiment started on April 16 the gas was held at an average of 35 percent and the treatment was continued for 20 hours.

The fruit was obtained direct from the grove, and after the treatment described it was shipped to the Arlington Experiment Farm, Rosslyn, Va., by ordinary express and was stored at 32° F. Notes were taken at various intervals on the development of disease. In the first experiment definite pitting on the control fruit ran about 50 percent higher than on the treated fruit; in the second experiment it ran about 90 percent higher. In both cases the treated fruit was more than 2 weeks behind the control fruit in developing a particular degree of definite pitting. In the first experiment there was little contrast between the treated and untreated fruit in the development of mild pitting, but in the second experiment it ran about 75 percent higher on the untreated fruit.

The reduction in pitting in the pony-refrigerator experiments was not as great as in the previous laboratory experiments, but the period of treatment was shorter and the percentage of gas lower. The 20-hour treatment with 35-percent carbon dioxide apparently gave better results than the 30-hour treatment with 20-percent carbon dioxide.

The flavor of the different lots of fruit was tested at various times and the treated fruit was found to be fully as good as the untreated fruit.

The results of the various carbon dioxide experiments seem to indicate that benefit can be obtained from the use of carbon dioxide as a prestorage treatment. Whether this benefit is due to a slowing down of metabolism, to the elimination of harmful products, or to some other cause has not been determined.

## PARAFFIN AND CELLOPHANE WRAPPERS

In a few experiments fruit was held in paraffin or in moisture-proof cellophane wrappers in comparison with other fruit in standard paper wrappers (table 9). With cellophane wrappers definite pitting was reduced in 6 out of 7 tests and mild pitting in 5 out of 7 tests, the average of the 7 results showing a reduction of about 43 percent for definite pitting and 36 percent for mild pitting. With paraffin wrappers both forms of pitting were reduced in 6 out of 8 tests, the average of the 8 results showing a reduction of about 36 percent for definite pitting and 40 percent for mild pitting.

Friend and Bach (5) have reported a similar reduction of pitting with paraffin wrappers. Their fruit was apparently held at 32° F., whereas the lots just mentioned were held at 36° or 40°.

## OILS AND WAXES

In other experiments mineral oils and various combinations of oils and waxes were used in wrappers or on the fruit. The results are shown in tables 10 and 11.

Wrappers that were treated heavily with mineral oil decreased definite pitting in 14 out of 17 tests, the average of the results showing a reduction of about 52 percent. The application of mineral oil to the fruit reduced definite pitting in 14 out of 15 tests, the average of the results showing a reduction of about 65 percent.

The heavily oiled wrappers decreased mild pitting in 12 out of 17 tests, the average of the results showing a reduction of about 57 percent. The application of oil to the fruit decreased mild pitting in 12 out of 15 tests, the average of the results showing a reduction of about 70 percent.

The wrappers used in the foregoing experiments were more heavily oiled than could be used commercially, and the writers are of the opinion that some of the pitting reported in the wrapper tests was really oil injury. Commercially oiled wrappers, used in a few tests, gave only a slight reduction in pitting and caused no injury.

TABLE 9.—*Effect of paraffin and cellophane wrappers on pitting of grapefruit stored at low temperatures*

Lot no.	Fruits under each condition	Storage temperature	Storage humidity <sup>1</sup>	Storage period	Peel showing—					
					Definite pitting			Mild pitting		
					With paraffin wrappers	With cellophane wrappers	With untreated wrappers	With paraffin wrappers	With cellophane wrappers	With untreated wrappers
	Number	°F.		Weeks	Percent	Percent	Percent	Percent	Percent	Percent
7	20	40	Low.....	8		2.40	6 1		1.20	2.90
8	8	36	High.....	6	0.35	.95	1 9	0 55	.42	.87
8	8	40	Low.....	10	.90	.90	1 2	2.50	3.20	6 10
9A	7	33	High.....	8	3 80	7.00	9 7	.74	1 50	1 70
9A	7	40	do.....	8	3 50		5.1	.40		.17
9A	7	40	Low.....	8	3.30	4.70	3.8	.28	.65	.74
9B	7	36	High.....	8	5.90	3.40	4.0	1.80	1.70	.34
9B	7	40	do.....	8	4.70		2.8	.65		1.00
9B	7	40	Low.....	8	4 30	5.40	13 2	.70	1 60	1.40

<sup>1</sup> "Low" indicates a relative humidity of 65 to 75 percent, "high", 85 to 90 percent.

TABLE 10.—*Effect of mineral oil on pitting of grapefruit*

Lot no	Fruits under each condition	Storage period	Storage temperature	Peel showing—					
				Definite pitting			Mild pitting		
				With mineral oil		Control	With mineral oil		Control
				On fruit	In wrap-pers		On fruit	In wrap-pers	
	Number	Weeks	°F.	Percent	Percent	Percent	Percent	Percent	Percent
2	26	9	32	-----	0.13	1.03	-----	0.03	0.92
3	21	9	32	-----	.17	.56	-----	.02	.81
6	8	10	36	2.30	2.10	3.60	0	0	.80
7	20	11	140	1.50	2.70	7.80	.95	2.00	2.70
8	8	10	36	0	0	.90	.78	.25	3.40
8	8	10	140	.90	.60	1.20	1.00	2.60	6.10
9B	16	8	32	.09	.27	.31	.30	.23	.16
9A	7	8	36	2.10	4.60	9.70	.90	2.70	1.70
9B	7	8	36	1.90	4.60	4.00	.74	0	.34
9A	7	8	40	1.70	4.10	5.10	.19	.40	.17
9B	7	8	40	2.00	6.00	2.80	.51	.40	1.00
9A	7	8	140	6.50	6.70	8.80	0	.28	.74
9B	7	8	140	4.80	5.40	13.20	.14	.25	1.40
10	8	7	40	.05	.75	2.60	0	.40	.08
10	8	7	140	.90	1.55	2.50	0	0	.28
11	8	7	140	-----	.42	2.20	-----	0	2.00
12	8	11	140	7.00	5.50	18.50	0	.40	.26
14	8	10	140	.60	-----	10.00	0	-----	.22

<sup>1</sup> With low humidity (65 to 75 percent).

TABLE 11.—*Effect of waxing on storage diseases of grapefruit*

Lot no	Fruits under each condition	Storage period	Storage temperature	Peel showing—								Fruit showing watery breakdown	
				Definite pitting		Mild pitting		Browning of oil glands		Scald		Fruit waxed	Control
				Fruit waxed	Control	Fruit waxed	Control	Fruit waxed	Control	Fruit waxed	Control		
	Number	Weeks	°F.	Percent	Percent	Percent	Percent	Percent	Percent	Percent	Percent	Percent	Percent
8	8	10	36	0	0.90	0.05	3.40	-----	-----	-----	-----	-----	-----
9A	7	8	140	.70	3.80	0	.74	-----	-----	-----	-----	-----	-----
9B	7	8	140	.77	13.20	0	1.40	-----	-----	-----	-----	-----	-----
10	16	7	40	.84	2.60	.18	.08	-----	-----	-----	-----	-----	-----
10	16	7	140	.47	2.50	.04	.28	-----	-----	-----	-----	-----	-----
11	8	7	140	.59	2.20	.15	2.00	-----	-----	-----	-----	-----	-----
12	8	11	140	4.30	18.50	.83	.26	-----	-----	-----	-----	-----	-----
13	8	13	140	.12	.50	.07	.12	-----	-----	-----	-----	-----	-----
14	8	10	140	.60	10.00	0	.22	-----	-----	-----	-----	-----	-----
15	9	9	36	.15	.78	0	.10	-----	-----	-----	-----	-----	-----
16	8	12	36	.05	3.60	0	.17	-----	-----	-----	-----	-----	-----
17	9	10	32	.05	.40	0	.13	0	0	2.5	2.30	0	0
17	9	10	36	.15	3.80	0	.30	0	0	0	0	0	0
18	24	12	32	0	.53	.30	.25	0	0	17.4	0	0	0
18	24	12	40	.05	.21	0	0	0	0	0	0	0	0
19	16	15	32	.04	.18	.67	.41	2.60	.70	0	0	0	0
19	16	15	36	.28	.71	.01	.10	.06	.02	0	0	0	0
20	24	13	32	.16	.30	.07	.30	11.80	4.50	0	0	0	0
20	24	13	36	.13	.50	.01	.13	0	.05	0	0	0	0
21	16	13	32	0	.22	.004	.05	0	0	0	0	43.8	12.5
22	16	13	36	1.00	4.40	.03	1.15	.48	.43	0	0	37.5	12.5
22	16	9	32	.15	.49	1.13	1.60	0	0	0	0	3.1	6.3
22	16	9	36	.13	.66	.12	.30	0	0	0	0	0	0
23	14	8	32	0	.02	.02	.01	0	0	12.1	.85	12.5	0
23	14	8	34	.06	.95	.004	.19	0	0	0	0	0	0
24	32	10	32	.02	.10	.14	.01	0	0	0	0	0	0
24	32	10	34	.17	.54	.05	.39	0	1.10	4.1	0	3.1	3.1
24	32	10	36	.05	.25	.05	.10	0	0	0	0	0	0
24	32	10	50	0	.004	.02	0	0	0	0	0	3.1	0
25	32	10	32	.02	.06	.48	1.80	0	0	5.2	1.30	21.9	3.1
25	32	10	34	.75	3.70	.50	.65	0	0	0	0	56.3	9.4
25	32	10	36	.71	3.10	.46	.72	0	0	0	0	21.9	9.4

<sup>1</sup> With low humidity (65 to 75 percent).

Fruit that was heavily waxed before storing developed less definite pitting than the controls in all of 32 cases where the disease was present in the controls, and the average reduction was about 82 percent (table 11). Heavy waxing resulted in the reduction of mild pitting in 26 out of a total of 33 tests, with an average reduction of about 63 percent. There was little contrast in the effect of waxing upon definite pitting at 32°, 34°, 36°, and 40° F. The reduction in mild pitting was greater at 36° than at 32° and 34°. The effect of the waxing upon both forms of pitting was more pronounced with low-humidity storage than with high-humidity storage.

The above contrasts in definite pitting were fully maintained when the fruit from 34°, 36°, or 40° F. was held for a week after removal from storage; i. e., more than five times as much definite pitting developed on the control fruit as on the waxed fruit in this period. In three-fourths of the lots stored at 32° the contrasts in definite pitting resulting from the waxing of the fruit were also maintained upon removal from storage, but there were a few definite exceptions.

The contrasts in mild pitting were fairly well maintained on the fruit from 36° and 40° F. upon removal from storage, but were largely lost on the fruit from 32° and 34°.

Browning of the oil glands, scald, and watery break-down were of sporadic occurrence, but there were several instances in which the last two of these diseases were very greatly increased as a result of waxing. Most of these instances were with fruit held at 32°. The modifying effect of temperature changes upon scald and break-down of waxed fruit is shown in table 8.

Experiments were made to determine the effects of delaying the application of wax for various periods after the fruit had been picked. Certain lots of fruit were waxed at Orlando at the time of packing, and comparable lots were similarly waxed 4 days later after shipment to Washington. In other experiments fruit was waxed after 1, 2, or 3 weeks' storage at 32° or 36° F. and compared with fruit that was waxed before storing at these temperatures. In all cases the results from delayed waxing were similar to those obtained from earlier applications.

The effect of waxing upon flavor was tested with various lots. There was seldom universal agreement among the persons making the tests, but the waxed fruit from 32° F. storage was usually rated lower than the control fruit from that temperature. The waxed fruit at 36° was often given as high rating as the control fruit and sometimes a higher rating. The fruit that had been heavily waxed seemed in some cases to be slightly less acid than the control fruit.

In most if not all of the above experiments the application of wax was heavier than would be practical under any commercial operation. In the later experiments an effort was made to secure a lighter waxing of the fruit. The lighter applications gave equally good results on the storage diseases, with little, if any, indication of any change in flavor.

The fruit that was waxed or wrapped in heavily oiled paper always looked fresher and had a better appearance than the control fruit. It also showed much less loss in weight. With high-humidity storage the control fruit usually showed a loss in weight of about 1 percent by the end of the first month, 2 percent by the end of the second month, and a much more rapid rate of loss with longer holding,



whereas the fruit that was waxed or wrapped in heavily oiled paper showed a rate of loss that was approximately 50 percent lower. With low-humidity storage the effect of waxing on loss of weight was much greater.

The waxes used in the above experiments consisted in all cases of a mixture of wax and oil combined in such proportion as to give a pliable product that could be readily rubbed on the fruit. Petrolatum and glycerin were occasionally included in the mixture, and water was sometimes added in an emulsion. Paraffin and carnauba were the usual waxes, and in some cases a small percentage of beeswax was added. Mineral oil was used in all instances. The writers have no best formula to recommend and are of the opinion that the modifying effects resulting from waxing are largely determined by the completeness of the covering.

In the experiments with lot 25, wax emulsions were tested in comparison with the wax mixtures reported above. The emulsions were prepared as follows:

A mixture consisting of 5 gallons of water, 0.9 pound of stearic acid, and 0.4 pound of triethanolamine was boiled gently with careful stirring until the acid was completely dissolved, giving a smooth soap solution. In a separate container 8.7 pounds of paraffin was melted over a hot water bath, care being taken that the temperature did not rise above 190° F. The melted paraffin was added to the boiling soap solution and stirred vigorously until an even dispersion of the wax was obtained; it was then stirred gently but continuously until cooled to room temperature.

Before being used, this emulsion was diluted, in some cases to 50, in some to 10, and in others to 1 percent of its original strength. The diluted emulsions were heated to 110° F. and the grapefruit was treated by rolling it around in the liquid for 1 to 3 minutes. In some cases the fruit was rubbed with a cloth after the emulsion treatment; in others it was dried before a fan.

In a few tests a modified emulsion was used to which beeswax and petrolatum had been added, and in another test ammonia was substituted for the triethanolamine.

The fruit that had received the various emulsion treatments was stored at 36° F. for comparison with similar untreated fruit and with fruit that had been coated with a mineral oil-wax mixture, as described above.

At the end of 10 weeks the waxed fruit showed a reduction in definite pitting of about 77 percent and a reduction in mild pitting of about 36 percent as compared with the control fruit, whereas the various emulsion treatments gave practically no reduction in either form of pitting. The waxed fruit showed a 30-percent reduction in loss of weight as compared with the control fruit, whereas the fruit that had received emulsion treatments showed an average of 6 percent reduction. There was no consistent variation in pitting nor in the loss in weight resulting from the different emulsion treatments.

#### EFFECT OF CHEMICAL VAPORS AND PEEL EXTRACTS

Grapefruit was found to respond to treatment with various chemical vapors. In the experiments reported in table 12 the fruit was held in 9-quart jars at room temperature during a 2-day treatment and later was stored at 32° or 36° F. The chemicals were dropped

into the jars from a pipette upon dry filter paper, and the jars were immediately closed.

The pitting produced by the vapor from the various chemicals was not always identical in appearance with that on the controls, yet the resemblance was close. It seems evident that many widely different chemicals are capable of producing pitting or of irritating the peel in such a manner that pitting follows.

In other experiments it was found that lemon oil applied in patterns to the peel of grapefruit occasionally produced injury in 32° and 36° F. storage. Steam-distillation extracts of grapefruit peel similarly applied produced injury resembling pitting in 8 out of 12 tests at both 32° and 40°. When grapefruit was held in closed jars at 70° and 3 to 6 cc of peel extract was introduced into the jars, slight but definite injury was evident in 4 days.

Puncturing the oil cells with a needle resulted in pitlike injuries, and in some instances there was a marginal browning that indicated injury from the oil released in puncturing.

Various attempts were made to produce similar effects by bruising the peel or by rubbing one grapefruit with the peel of another. In most cases the results were negative, but distinctly positive results were obtained with fruit from lot 25. The fruit had been in low-temperature storage several weeks before the experiment was started but had been warmed to room temperature before the treatments were given. Oil and juice were squeezed out of fresh grapefruit peel and rubbed on marked areas on other grapefruit, and the fruit was then stored at 31°, 32°, and 36° F. After 1 month's storage 18 out of a total of 48 treated areas showed injury, whereas the untreated areas remained free from injury. There was no significant contrast in the results at the different temperatures.

TABLE 12.—*Effect of various chemical vapors upon grapefruit*

Chemical	Quantity	Effect of treatment
	Cc	
Ammonia.....	0.8 to 1.7.....	Pitting greatly increased. Fruit more yellow in some cases
Do.....	0.3 to 0.7.....	No contrast in pitting Fruit more yellow in some cases
Acetic acid.....	0.5 to 3.0.....	Pitting greatly increased; color decreased.
Do.....	0.3.....	No evident effect.
Alcohol, 95-percent.....	3 to 5.....	Do.
Acetaldehyde.....	0.4 to 0.5.....	Pitting greatly increased.
Do.....	0.3.....	No evident effect.
Formalin.....	0.3 to 1.7.....	Pitting greatly increased.
Petroleum ether.....	2.5 to 3.....	Pitting worse than in control.
Do.....	1 to 2.....	No evident effect.

The injury reported in table 12 was largely in the nature of definite pitting; that produced by lemon oil and grapefruit extracts was almost entirely of the oleocellosis type (fig. 8) but shading off in some instances into various degrees of pitting.

#### DISCUSSION

The foregoing experiments seem to give abundant proof that the seriousness of the storage diseases of grapefruit may be greatly modified by after-harvest treatment, yet the results have not always been consistent. Great care was taken in selecting and sampling the fruit

for treatment but it was found extremely difficult to make certain that the lots to be compared were physiologically similar. As grapefruit is seen in the picking crate or in the packing house there is considerable variation in the thickness of the peel and in the smoothness of its surface, and this physical variation is apparently indicative of differences in physiological response. The maturity of the fruit is also a disturbing factor from the standpoint of accurate experimentation. The grapefruit tree has a prolonged season of blooming, and fruits hanging side by side with no difference in appearance may be months apart in actual age. Fruit that is picked in the fall is likely to be from the early bloom and fairly uniform as to age, whereas fruit picked in the spring may be from the early, intermediate, or late bloom and represents a wide variation in age and probably in senility. From the standpoint of accurate experimentation the early pickings should be more desirable, but from the practical point of view it is the later pickings that should be studied because it is the late fruit that is likely to be stored.

With this background of variability it is not surprising that the results are not always consistent. It is not only possible that the individuals of a particular lot of fruit may have a wide variation in physiological condition but that the whole lot may be physiologically different from another lot, especially if grown in a different section or picked in a different season.

In spite of the variability in material and in results, certain principles regarding the storage behavior of grapefruit appear to have been established. Pitting has been decidedly increased by low humidity in the storage atmosphere, by the placing of soil or other foreign material inside the wrappers, and by the impregnation of the wrappers with glycerin. It has been decreased by high humidity in the storage atmosphere, by the use of cellophane, paraffin, or heavily oiled wrappers, and by coating the fruit with various oil-and-wax mixtures. All of these agencies have had a modifying influence upon the loss of moisture from the fruit, and it is the tentative opinion of the writers that this may be the main cause of their harmful or beneficial effects. This does not mean that pitting is to be considered a mere drying out of the peel. It is entirely distinct from shriveling and apparently is due to the effect that loss of moisture has upon enzyme or other physiological activities. A recent publication by Lauritzen and Balch (10) is significant in this connection. They found that the inversion of sucrose in the pile of sugarcane is intimately associated with the loss of moisture, increasing with the drying out of the cane. It seems probable in the present case that high humidity tends to delay some phase of destructive metabolism in the peel.

Nelson (11) has recently concluded that pitting of grapefruit is probably a manifestation of suboxidation. He was able to produce typical symptoms by holding the fruit in nitrogen at room temperature for 4 to 7 days. At temperatures of 31° to 33° and 38° to 42° F., pitting developed in 17 to 22 days when there was no deficiency of oxygen in the storage atmosphere. Two hypotheses were offered as possible explanations for the occurrence of the disease at low temperatures; one, that the oxidizing system is affected by low temperature to such an extent that there is a slow accumulation of toxic materials; the other, that low temperature may cause a preponderance of hydrolytic activity resulting in excessive splitting of substances like glucosides

and the consequent accumulation of materials that poison the protoplasm.

The data of the present paper give considerable support to Nelson's hypotheses and conclusions, yet there is much that can be interpreted otherwise.

Coating apples with oils and waxes or wrapping them in oiled paper is known to increase the carbon dioxide content and decrease the oxygen content of the internal atmosphere and the same thing is probably true for grapefruit, yet treatments of this type, as well as prestorage treatment with carbon dioxide, have resulted in a decrease in pitting. These facts do not seem to be in harmony with the theory of suboxidation yet they do not preclude the possibility that, other things being equal, a low oxygen supply may favor the development of the disease. Soft scald of apples has been classed as a suboxidation disease and, like pitting of grapefruit, is decreased by coating the fruit with wax or by prestorage exposure to high temperature or to carbon dioxide gas(2). This complete parallelism under such diverse treatments between diseases of two fruits so widely separated as the grapefruit and the apple makes a basis for interesting speculation, and would seem to form a background in the search for a fundamental cause, viz, some condition of metabolism that might be similarly modified by the various treatments.

Pitting of grapefruit and soft scald of apples are similar also in having definite temperature limitations, but their critical temperatures are different. Soft scald is most pronounced at a storage temperature of 30° to 32° F., and is largely eliminated by holding the fruit at 36° to 38°, whereas definite pitting is much worse at 36° than at 32°, but can be largely prevented by storing at a temperature of 45° to 50°. Whatever similarity there may be in the fundamental causes of the two diseases, it is evident that there are factors involved for which the temperature requirements are distinctly different.

Scald and watery break-down of grapefruit can be more readily classed as suboxidation diseases than pitting. They are increased by coating the fruit with wax and by subjecting it to high temperatures prior to storage, and their occurrence is especially favored by storage at 30° to 32° F.

Certain phases of the present report have important practical significance. Perhaps the most significant fact is the emphasis given to humidity. It has been commonly recognized that a high humidity is desirable in the storage rooms for grapefruit, but it is doubtful whether its extreme importance has been fully realized.

Rubbing a coat of wax (a mixture of wax and oil) on the fruit has greatly decreased pitting and decreased the loss in weight, but attempts to secure a satisfactory coating by means of wax emulsions have proved a failure under the conditions of the experiment (p. 346).

It has been found that grapefruit can be exposed to low temperatures for 2 weeks and sometimes for 3 or 4 weeks without the tendency toward physiological disorders becoming irreversibly established. Applications of wax at the end of 2 or 3 weeks' storage have been practically as effective in controlling pitting as have similar treatments before storage. Moving the fruit from 32° or 36° to 70° F. for 1 day or to 50° for 1 week at the end of 2 weeks' storage resulted in a great decrease in pitting, and moving it to 50° permanently at the end of 2 weeks resulted in practically complete elimination of pitting.

These results need further checking, but if it can be definitely established that grapefruit can be held for at least 2 weeks at a low temperature without developing a permanent tendency toward pitting it should make possible greater freedom in methods of shipment and storage, especially with fruit from sections where decay is a serious problem.

The knowledge that pitting, scald, and watery break-down are definitely decreased by removal to higher temperatures for short periods at the end of 1 or 2 weeks' storage at 32° or 36° might be of value at times in planning the transfer of fruit from shipping point to market-terminal storage.

#### SUMMARY

Descriptions are given for two forms of pitting and for scald, oleocellosis, browning of the oil glands, and watery break-down of grapefruit.

Bacteria were readily isolated from pitted tissue and certain forms were found capable of producing pits when inoculated into grapefruit peel but various modifying factors indicate that the disease is not primarily of bacterial origin.

Mild pitting was more pronounced and definite pitting about five times as bad at low as at high humidity. Both were increased by placing a small quantity of soil, sand, or charcoal inside the wrapper, or by impregnating the wrappers with glycerin, and all of these treatments resulted also in a decided increase in the loss of moisture.

Definite pitting was much more serious at 36° and 40° F. than at 32°, whereas mild pitting was somewhat worse at 32° than at 36° and 40°. Both were practically eliminated by holding the fruit at 50° F. Scald and watery break-down were worse at 30° and 32° than at 36° and 40°.

Holding grapefruit at temperatures of 60° to 75° F. before storing it at 36° resulted in a definite decrease in pitting, but holding the fruit at 50° before storing it at lower temperatures had no effect upon the later development of pitting. Prestorage heating for 17 to 22 hours at a temperature of about 100° resulted in a very great decrease in pitting in fruit stored at 36° or 40°, but in many cases gave a definite increase in fruit stored at 32°. Scald was much worse on heated fruit, especially that subsequently held at 32° storage.

Removing the fruit from room temperature for 1 day at the end of 1 week and again at the end of 2 weeks or moving the fruit to 50° F. for 1 week at the end of 2 weeks resulted in a decided reduction in both definite and mild pitting and also in scald and watery break-down. Removing the fruit to 50° permanently at the end of 1 or 2 weeks' storage at a lower temperature resulted in the practical elimination of physiological disorders.

Exposing grapefruit for 20 to 48 hours to atmospheres containing 20 to 45 percent of carbon dioxide before placing it in low-temperature storage resulted in a definite decrease in the later development of pitting, the best results being obtained with the longer treatments and the higher percentages of gas.

Storing the fruit in paraffin or cellophane wrappers gave a reduction in pitting similar to that obtained with initial carbon dioxide treatments.

Excessively oiled wrappers gave a much greater reduction in pitting than paraffin or cellophane wrappers.

Coating the fruit with mixtures of mineral oil and wax resulted in a much better control of pitting than any type of wrapper used but sometimes caused an increase in scald and watery break-down, especially with heavy coating and 32° F. storage.

Fruit held at 50° F. soon developed a very high percentage of stem-end rot, whereas fruit held at 40° or lower usually showed no decay at the end of 8 weeks' storage.

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# EXTENT OF VERTICAL MIGRATION OF HORSE STRONGYLE LARVAE IN SOILS OF DIFFERENT TYPES<sup>1</sup>

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## INTRODUCTION

Plowing under of soil infested with eggs and larvae of livestock parasites is generally considered a useful prophylactic measure against worm parasites. However, in 1932, Hall (2)<sup>2</sup> called attention to the lack of adequate information concerning the ability of nematode larvae to migrate vertically in soil, and emphasized the need for experimental investigations on this subject. The experiments described in this paper were undertaken to obtain information on the extent to which the vertical migration of horse strongyle larvae in soil occurs. Such information is closely related to the value of plowing under as a control measure for horse strongyles.

## REVIEW OF LITERATURE

Since Ransom's (5) investigation on the life history of *Haemonchus contortus*, published in 1906, various investigators have noted a tendency on the part of infective strongyle larvae to climb up the walls of glass containers when a film of moisture was present on the sides of the culture vessels. Nöller and Schmid (4) reported that horse strongyle larvae commonly show this tendency. Enigk (1) stated that these larvae climb up stable walls which have been treated with milk of lime, provided the boards are moist. Wetzel's comment on Hüber's paper (3, p. 710) noted that horse strongyle larvae climb up grass blades in wet or damp weather and return to feces or soil as sunshine dries the grass. Schwartz, Imes, and Wright (7) make the following statement regarding the vertical migration, on grass, of larvae of the large horse strongyles:

When the air is sufficiently moist so that the grass becomes covered with a film of moisture, as happens in times of rain, dew, or fog, the larvae migrate up the grass blades and this brings them to a favorable situation to be swallowed by horses while grazing.

This statement was based on observations by Schwartz<sup>3</sup> who, by means of the Baermann apparatus, isolated horse strongyle larvae from those portions of blades of grass which had no contact with the soil, the larvae being especially abundant on the grass after dews or rains. Similar observations have been reported in connection with the behavior of larvae of other strongylid nematodes.

So far as is indicated by examination of the literature dealing with the behavior of horse strongyle larvae, the vertical migration of these larvae in soil has not been previously investigated. In fact, investi-

<sup>1</sup> Received for publication Oct. 2, 1935; issued April 1936.

<sup>2</sup> Reference is made by number (italic) to Literature Cited, p. 361.

<sup>3</sup> Personal communication to the author.



gations concerning the vertical migration in soil of the larvae of strongyles of domestic animals have been confined to only a few species. Ross and Kauzal (6) recently reported experiments which indicate that the infective larvae of *Stephanurus dentatus*, a species parasitic in swine but reported once from an equine, showed very little tendency to reach the surface of mud when buried to depths varying from about 5 to 7.5 cm; Spindler (9) buried several hundred infective larvae of *S. dentatus* beneath 2.5 inches of moist soil in each of 12 tin cans; only 9 larvae were recovered from the surface soil in the cans during the course of daily examinations over a period of 3 weeks. Spindler did not find larvae of this parasite in six weekly examinations of the surface soil from a freshly plowed pasture which had harbored numerous *Stephanurus* larvae before the land was plowed. The same investigator (8) obtained a similar result in an extensive field investigation involving the vertical migration in soil of the larvae of *Oesophagostomum* of swine.

#### METHODS USED IN THIS INVESTIGATION

Freshly passed horse feces, uncontaminated with bedding or soil, were collected and cultured in glass containers at room temperature (69° to 76° F.). Infective larvae were obtained from these feces by means of the Baermann isolation apparatus.

Soils varying as much as possible in type were obtained near Beltsville, Md. Screened soil of the desired type was rendered helminthologically sterile by heating it in open pans until all of it reached a temperature of at least 156° F. In some tests unheated soil was used; this soil was taken from localities to which, so far as is known, horses had had no access. Moreover, before unsterilized soil was used in the tests, samples were placed in the Baermann apparatus and no horse strongyle larvae were found in them.

The sterilized and tested unsterilized soils were moistened with water and firmly packed into containers, usually wooden boxes. The boxes either had wooden bottoms in which a number of holes were bored, or bottoms of fine wire screen in order that water might pass downward through the soil in a normal manner. Excavations about 1.5 inches in diameter and of the desired depths were made in the center of the soil in the containers.

Except in one experiment, no. 8, larvae which had attained the infective stage not more than 1 or 2 weeks previous to the commencement of the experiment, suspended in a few cubic centimeters of water, were placed at the bottom of the excavations by means of a long glass pipette. A few minutes were allowed to permit absorption of the water by the soil before the excavations were refilled with the previously removed soil, which was carefully compressed. In all the experiments except no. 8, the larvae placed in the soil were counted, a dilution method being employed for this purpose.

In making the vertical-migration studies, 10 experiments were carried out. Experiments 1 and 3 were performed in the laboratory. Glass tubes about 8 inches high and 6 inches in diameter, open at both ends and resting on tin plates, were used as containers for the soil. After the larvae had been buried, lids were placed over the mouths of the tubes and the preparations were placed in a dark cupboard where they remained throughout the duration of the experiment.

Experiments 2, 5, 7, 8, and 9 were made outdoors on small fenced plots in the vicinity of Beltsville, Md. In experiments 2, 7, and 9, wooden boxes about 13 inches square and 8 inches deep were used as containers for the soil. The boxes were sunk in sandy soil in such a manner that their upper edges protruded 1 inch above the surface of the ground, and the level of the soil in the boxes was about the same as that of the surrounding soil. In experiment 5, boxes about 17 inches square, having a depth of either 4 or 8 inches, were used. They were placed in shallow excavations made in sandy soil. In experiment 8, four bottomless wooden frames, about 15 inches square and 10 inches deep, were sunk in sandy soil to such a depth that their upper edges protruded about 1 inch above the surface. The soil was completely removed from within the frames and replaced with tested sand. Pellets of horse feces containing large numbers of infective horse strongyle larvae were buried at the bottom of excavations, about 3 inches in diameter, prepared in the soil within each frame.

Experiments 4, 6, and 10 were carried on inside a small wooden shelter located on a shaded lot. The shelter afforded complete protection from rain, and the temperature within the shelter varied in accordance with the temperature of the outside air. In each experiment, two wooden boxes about 13 inches square, one of which was 4 inches and the other 8 inches deep, were used as containers for the soil. Tall metal cylinders about 12 inches in diameter were pressed into the soil to a depth of about one-half inch to prevent larvae from migrating out of the boxes as a result of possible lateral movement in the surface layer. A large metal box, about 6 feet long, 4 feet wide, and 15 inches deep, was filled with moist soil, and the wooden boxes were sunk in this soil in such a manner that the level of the soil within and without the wooden boxes was approximately the same. Water not only was sprinkled on the surface of the soil in the wooden boxes from time to time but also was poured into a short metal pipe located in one corner of each box.

In all experiments in the laboratory or under shelter, the soil was kept moist by the addition of water from time to time. The variation in the temperature of the laboratory has been indicated previously. In the outdoor experiments in which no shelter was provided, water was not added to the soil in the boxes after the larvae were buried. Precipitation invariably occurred during the period in which the experiments were in progress.

At the expiration of the desired interval, the entire surface layer of the soil in the container was scraped off with a spoon or spatula to a depth of about one-eighth of an inch to collect larvae that had migrated to the surface. The scrapings were placed in the Baermann apparatus, and after 24 hours or longer the fluid was withdrawn from the bottom of the rubber tubing and the larvae recovered were counted.

## EXPERIMENTAL DATA

### VERTICAL MIGRATION AS AFFECTED BY TYPE OF SOIL

The data pertaining to the vertical-migration experiments with different types of soil (table 1), show that in from 19 to 53 days there was extremely little migration of horse strongyle larvae to the surface of clay soil from depths of 1 to 6 inches. In heavy sandy loam to clay

TABLE 1.—Results of experiments on vertical migration of horse strongyle larvae in types of soil indicated <sup>1</sup>

## HEAVY SOIL

Soil type	Experiment no.	Location of experiment	Con-tainer no.	Approximate number of larvae buried	Depth of burial of larvae	Date of burial	Time from burial of larvae to examination of surface soil	Larvae recovered from surface soil		Meteorological data <sup>2</sup>	
								Number	Percent	Mean temperature	Total precipitation
Clay	1	Indoor	{	28,000	1	Jan 25, 1933	19	7	0.02	69	3.09
				28,000	2	do.	19	0	0	69	3.09
				28,000	2	do.	53	0	0	69	3.09
	2	Outdoor	{	( <sup>3</sup> )	2	May 16, 1934	23	0	0	69	3.09
				45,000	3	do.	23	2	0.004	69	3.09
				45,000	6	do.	23	0	0	69	3.09

## MODERATELY HEAVY SOIL

Heavy sandy loam to clay loam	3	Indoor	{	50,000	1	Nov. 16, 1932	26	10,500	21.0		
				58,000	2	Nov. 15, 1932	27	1,500	3.9		
Sandy clay loam	4	Outdoor, under shelter	{	67,000	4	do.	28	2	0.003		
				37,000	5	do.	28	0	0		
				40,000	2	July 25, 1934	44	1,427	3.5	74.3	
				40,000	5.5	do.	37	69	.17	73	

## MODERATELY LIGHT SOIL

Sandy loam	5	Outdoor	{	12,000	1	Jan. 30, 1933	63	25	.2	41	6.09
				20,000	2	Jan. 26, 1933	67	75	.37	41	6.87
Do.	6	Outdoor, under shelter	{	70,000	3	Feb. 6, 1933	60	250	.36	42	6.62
				16,000	5	Feb. 21, 1933	62	12	.07	47	8.13
				93,500	6	July 27, 1933	41	85	.09	76	10.90
				40,000	2	July 25, 1934	44	407	1.0	74.3	
Sandy light clay loam	7	Outdoor	{	40,000	5.5	do.	37	11	.027	75	
				45,000	2	May 5, 1934	23	887	1.9	67	2.72
				45,000	3	May 3, 1934	25	550	1.9	67	3.54
				45,000	6	do.	25		1.2	67	3.54

## LIGHT SOIL

Coarse sand	8	Outdoor	{	1	(1)	13	Sept. 20, 1933	27	7,190	---	58.6	3.87
				2	(1)	15	do.	27	12,704	---	58.6	3.87
				3	(1)	16	do.	28	8,031	---	58	3.87
	9	do.	{	4	(1)	18	do.	28	6,920	---	58	3.87
				1	45,000	2	May 3, 1934	28	22	---	69	3.67
				2	45,000	3	do.	28	232	.05	69	3.67
	10	Outdoor, under shelter	{	3	45,000	6	do.	28	53	.12	69	3.67
				1	40,000	2.5	July 25, 1934	44	277	.69	74.3	---
				2	40,000	5	do.	37	42	.10	75	---
				2	40,000	5	do.	37	42	.10	75	---

<sup>1</sup> Soil designations by J. E. Lapham of the Bureau of Chemistry and Soils, U. S. Department of Agriculture.

<sup>2</sup> Taken from reports of the Washington, D. C., station of the Weather Bureau, U. S. Department of Agriculture, located approximately 13 miles southwest of Beltsville, Md.

<sup>3</sup> Number undetermined but exceeded 45,000.

<sup>4</sup> Fecal pellets containing larvae were buried. It was impractical to determine the number of larvae present.

<sup>5</sup> The depth given is that of the column of sand above the uppermost surface of the fecal pellets.

loam and sandy clay loam, very few of the larvae buried to depths of 4 to 5.5 inches reached the surface in intervals ranging from 28 to 37 days; from depths of 1 to 2.5 inches, however, appreciable numbers of larvae reached the surface in from 26 to 44 days. In sandy loam and sandy light clay loam, small percentages reached the surface from depths of 1 to 6 inches in from 23 to 67 days. Likewise, small percentages reached the surface of coarse sand from depths of 2 to 6 inches in from 28 to 44 days. The results of experiment 8, although the larvae buried were uncoun ted, indicate that considerable numbers reached the surface of coarse sand in 1 month when small quantities of horse feces were buried from 3 to 8 inches beneath the surface. In general the individual experiments show that the percentage of larvae which reached the surface varied inversely with the depth of burial.

#### VERTICAL MIGRATION AS AFFECTED BY LATERAL SUBSURFACE MIGRATION

Since the soil containers used in the vertical-migration experiments were very limited in size, it was desirable to know whether the results might have been affected by lateral migration of the larvae beneath the soil surface to the edges of the containers, followed by ascent along the surface of the containers. Two experiments were performed to determine the extent of such migration, sandy light clay loam being used in each experiment.

In the first of these experiments, a covered wooden box  $7\frac{1}{4}$  inches long, 6 inches wide, and  $6\frac{1}{2}$  inches deep, was filled with soil. The box was so constructed that sections of the sides could be withdrawn without disturbing the position of the moist soil. On March 24, 88,000 larvae were buried to a depth of 4 inches in the center of the soil. The distance from the sides of the rectangular excavation, in which the larvae were placed, to the sides of the box was  $2\frac{1}{4}$  inches. On April 10, 17 days after the experiment began, 66,500 larvae were recovered from the surface soil. Of these larvae, 143 were in the soil directly above the area where the larvae were originally buried, 904 were in the surface soil within a distance of about 1 inch from the central rectangular area, and the remainder were recovered from the area extending from this point to the sides and ends of the box. Sections of one side of the box were removed, and 42 larvae were found in the soil of the lateral surface at an average distance of  $3\frac{1}{2}$  inches from the top; 16 larvae were found at an average distance of  $1\frac{1}{4}$  inches from the top. On the opposite side, 25 larvae were found in the lateral surface soil at a distance of about 2 inches from the top. Larvae were isolated also from the surface of the wooden sections removed from the box.

In the second experiment a wooden box about 17 inches square and 6 inches deep, resting on a thin layer of moist soil in a large metal tray, was filled with soil. About 94,000 larvae were buried in a central excavation 1 inch in diameter and  $2\frac{1}{2}$  inches deep. A metal cylinder  $2\frac{1}{2}$  inches in diameter was placed directly over the spot beneath which the larvae were buried; the lower edge of the cylinder was pressed into the soil to a depth of about one-half inch. In a similar manner, tall metal cylinders, 5, 8, and 10 inches in diameter, were placed concentrically about the small cylinder. Barriers were thus erected to prevent the possibility of migration of larvae laterally on the surface of the soil, the arrangement permitting a determination and comparison

of the number of larvae reaching the surface in each of the five separate areas so produced. After 19 days the surface soil from the area extending from the edges of the largest cylinder to the sides of the box was removed, and after 21 days the surface soil from each of the remaining areas was removed, each sample being separately examined. The results are shown in table 2.

TABLE 2.—*Results of experiment to determine effect of lateral subsurface migration on vertical migration of horse strongyle larvae*

Area no.	Larvae recovered <sup>1</sup> —		Area no.	Larvae recovered <sup>1</sup> —	
	From surface soil	Per square inch of surface		From surface soil	Per square inch of surface
	<i>Number</i>	<i>Number</i>		<i>Number</i>	<i>Number</i>
1 (center).....	3,468	707	4.....	2	0.07
2.....	3,043	206	5 (outer).....	8	.04
3.....	18	.58			

<sup>1</sup> The total number of larvae buried was 94,000.

The data from the two experiments just described indicate that lateral migration beneath the soil surface occurs during the ascent of the larvae to the surface layer. In reaching the surface from a depth of  $2\frac{1}{2}$  inches, in the second experiment, a few larvae migrated laterally  $4\frac{1}{2}$  inches or more, but the vast majority did not migrate more than 2 inches laterally during their ascent. In this experiment approximately 7 percent of the buried larvae reached the surface from a depth of  $2\frac{1}{2}$  inches in from 19 to 21 days. In the first experiment, about 75 percent of the buried larvae reached the surface in 17 days.

## DISCUSSION

It is difficult to compare the writer's results with those of Ross and Kauzal (6) and Spindler (9) in their experiments with larvae of *Stephanurus dentatus*, since these investigators did not definitely mention the type of soil used. Spindler (8, 9) concluded from his field studies that plowing the soil under and later growing a crop on it frees the land of *Stephanurus* and *Oesophagostomum* larvae; this procedure is regarded by Spindler as an effective control measure for these parasites.

In the writer's experiments, horse strongyle larvae migrated vertically in coarse sand, sandy loam, sandy light clay loam, sandy clay loam, and heavy sandy loam to clay loam, distances equivalent to those attained by shallow to average plowing (from 4 to 6 inches). However, from such depths only from 0.003 to 1.2 percent of the buried larvae reached the surface of these soils in from about 3 weeks to 2 months. If it is assumed that the rates of migration indicated by the experiments for the periods mentioned remain approximately constant, it is evident that only a small percentage of the larvae actually buried 4 inches or deeper would reach the surface of the soil in the course of a year. However, the writer's experiments showed also that a considerable percentage of buried larvae quickly reached the surface from shallow depths, particularly in loam soils. It is evident, therefore, that although plowing under of infested soil

undoubtedly possesses considerable value as a control measure for strongylidosis in the horse, the efficacy of this control measure has definite limitations, being affected by the depth to which the larvae are buried and the type of soil in which they are present. It is indicated by the experiments that if larvae are buried in clay soil by plowing, very few will subsequently reach the surface.

The experiment described on page 358, in which a very large percentage of buried larvae quickly reached the surface of sandy light clay loam soil from a depth of 4 inches, may appear to present a result inconsistent with certain of the above statements. The succeeding experiment (p. 358) suggests, however, that the larvae migrated laterally beneath the surface of the soil to the sides of the box and ascended these moist surfaces to the upper soil layer. Enigk (1), as already stated, has shown that horse strongyle larvae migrate vertically along moist wooden surfaces. Such upward migration probably occurs on plant roots. The results of these two experiments indicate that lateral subsurface migration was not a factor seriously influencing the results of the writer's experiments on vertical migration.

#### SUMMARY AND CONCLUSIONS

Experiments performed indoors and outdoors indicate that there is practically no vertical migration of horse strongyle larvae in clay soil.

Experiments made indoors and outdoors under shelter showed that extremely small percentages of buried larvae reached the surface of heavy sandy loam and sandy clay loam from depths of 4 to 5.5 inches in from about 4 to 6 weeks. During similar periods, 3.5 percent or more of buried larvae reached the surface of these soils from depths of 1, 2, and 2.5 inches.

Both indoor and outdoor experiments involving sandy loam and sandy light clay loam soils showed that from 0.027 to 7 percent of buried larvae reached the surface from depths of 1 to 6 inches in from 19 to 67 days; in general, the percentage of larvae reaching the surface varied inversely with the depth to which they were buried. An experiment in which about 75 percent of the buried larvae reached the surface in less than 3 weeks is excepted in these statements because the data indicate that the larvae ascended the moist wooden sides of the small box in which the soil was kept. Larvae probably could ascend plant roots in the same way.

Small percentages of larvae reached the surface of coarse sand from depths of from 2 to 6 inches in from 28 to 44 days. In a non-quantitative experiment involving coarse sand, appreciable numbers of larvae reached the surface from depths as great as 8 inches in about 4 weeks.

Experiments involving sandy light clay loam showed that during vertical ascent to the surface some larvae migrated laterally for short distances beneath the soil surface.

Deep plowing under of infested soil should be of some value for the control of strongyle parasitism in horses, but the efficacy of this control measure is subject to definite limitations, particularly in respect to the depth at which the larvae are buried by plowing and the type of soil in which they are present. Clay soil presents an almost complete barrier to the vertical movement of the larvae.

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# ZINC AND OTHER MINERAL CONSTITUENTS IN RELATION TO THE ROSETTE DISEASE OF PECAN TREES<sup>1</sup>

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## INTRODUCTION AND REVIEW OF LITERATURE

Rosette is a physiological disease of pecan trees. Symptoms are variable but usually include a mottled chlorosis and crinkling of newer tipmost leaves and shortening of the internodes of new shoot growth. Affected leaflets do not attain normal size and are frequently malformed. A necrosis and abscission of leaflets and entire leaves may follow. The resulting leafless shoots frequently die back. New growth arises below the dead area. This usually becomes affected in the same or subsequent growing season. The tree becomes misshapen and worthless, though it seldom dies outright. The disease occurs widely throughout the Southern States. Its presence was reported in Arizona in 1911.

In studies of pecan rosette in Arizona previously reported (8),<sup>3</sup> it was found that both strongly and weakly vegetative trees are affected. This fact was interpreted as meaning that factors responsible for rosette are somewhat independent of those governing length of vegetative extension. Amount and length of growth in plants have been reported by many workers to be closely related to carbohydrate and nitrogen nutrition in the plant. If imperfections in nutrition were responsible for rosette, they would be of a mineral rather than of an organic origin. Some ions and elements are not readily available in alkaline soils. Occurrence of rosette is common on, but not limited to, alkaline soils.

Similar chloroses and malformations in other plants have been induced by growing the plants in a nutrient deficient in certain ions (4, 11) or, where occurring in the field, have been corrected by application of minerals not supplied in ordinary fertilizers (2, 10, 21).

As organic nutrition is unlikely as a cause, and some ions may not be satisfactorily available in the alkaline soils of Arizona, and also since rosetted trees display symptoms similar to those in other plants which were corrected by the addition of certain ions, the conclusion was that rosette might be due to a mineral deficiency. To test for a mineral deficiency, the most effective procedure is to use many differing elements and ions and to apply them singly, not to the soil where fixation might render them unavailable to the tree or where a response of the tree to them might be slow or indefinite, but rather to apply them directly to the aboveground parts of the tree where their effect might be readily noted. This procedure revealed that pecan rosette yielded to treatment with zinc (8). A

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<sup>2</sup> The writer is indebted to the members of the Department of Agricultural Chemistry, University of Arizona, who kindly permitted the use of their laboratory and equipment for conducting the analyses described herein. Especially is gratitude extended to Dr. R. A. Greene, who gave counsel throughout the analytical procedure.

<sup>3</sup> Reference is made by number (italic) to Literature Cited, p. 375

similar method was used by Alben et al. (1). Other workers have reported a curative action of zinc (6).

The various data leave little doubt but that pecan rosette yields to treatment with zinc. Before this can be finally concluded, a strictly chemically pure form of zinc must be used. As far as the writer is aware no worker has yet used such a material. The fact that similar symptoms in peach, plum, apricot, grape, walnut, and orange in California (5), cherry in Oregon (15), citrus in Arizona (7), and tung, citrus, and corn in Florida (17) respond similarly to zinc indicate a common causal relation of the various diseases.

Rosetted pecan trees in Arizona respond readily to the application of zinc sulphate to the soil around the trees (9), and are now being treated satisfactorily in that way. In the alkaline soils of Arizona, pecan trees are not injured to any appreciable extent by a toxic action of zinc sulphate in the soil. Similar treatment of citrus mottleleaf is not practical here because of the toxic action of zinc on the trees, as has been reported by Parker (18) in California. Further information on the occurrence and availability of zinc in the soil may lead to the devising of cultural methods to make zinc more available and thereby effect a remedy without the actual addition of zinc. The present paper reports experiments in which zinc was used in treating pecan trees affected with rosette.

#### THE PROBLEM

With the recognition that pecan rosette is in some way associated with zinc, interest focuses upon the occurrence and action of zinc in the plant, particularly in view of certain commonly observed phenomena. The top of the tree is most susceptible to rosette. Is this because of less zinc in the tissue? Similarly, why may one limb be healthy and another on the same tree be affected? Why may one tree be affected in an otherwise healthy orchard, and vice versa? Why do trees healthy for many years suddenly become affected or trees diseased for many years become healthy with or without change in cultural treatment? Is this because of a change in the total amount of zinc in the tissue or is it a question of availability of zinc for physiological use? Is the availability of zinc modified by the presence of other elements or ions in the tissue? To gain initial information upon the occurrence, distribution, movement and availability of zinc, analyses were made of pecan tissue for ash, silica, calcium, magnesium, iron, copper, and zinc in 1933, and for ash and zinc in 1934.

#### EXPERIMENTAL METHODS AND MATERIALS

##### SEASON OF 1933

An 8-year-old orchard of Burkett pecan trees growing near Tucson, where rosette has been generally severe, was used. One previous sampling of the irrigation water revealed an absence of zinc (8). The soil had a pH value of approximately 8 and contained about 200 p. p. m. of total soluble salt. Most of the trees in the orchard were small and misshapen from rosette, but several, including those sampled, were of ordinary size for their age. All displayed evidence of having had rosette in some previous years. The healthy untreated tree used for sampling was apparently not far removed from a rosetted

condition, for while it showed no symptoms at time of sampling, it displayed mild chlorosis by late August. This may mean that if the present studies measured factors related to the cause of rosette, the widest possible extremes between healthy and affected tissue were not represented in these samples.

On June 22 four trees were selected. Two of these had chlorotic leaves in the topmost parts, but there was no necrosis of shoots. The other two trees showed no symptoms of rosette. One diseased and

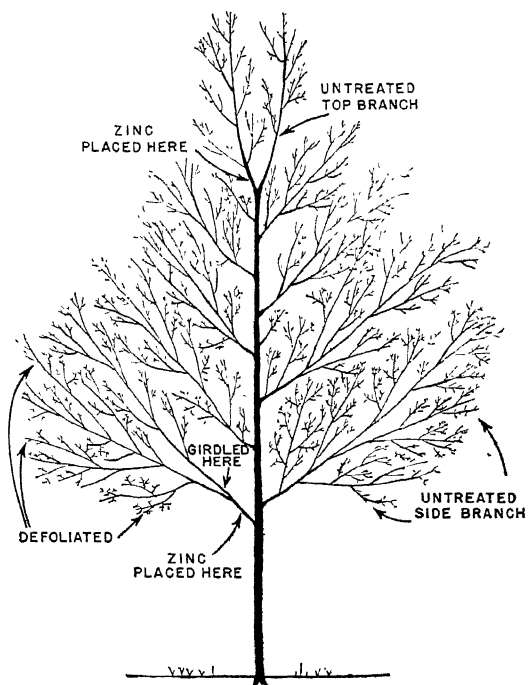


FIGURE 1—Diagram of tree given special treatment as described in the text. The applications of zinc, the defoliation, and the girdling were done on July 14. Samples for analysis were collected on August 18. The defoliation was not repeated so that new growth had its full complement of leaves when sampled.

one healthy tree was treated by placing approximately 30 g of commercial zinc sulphate ( $\text{ZnSO}_4 \cdot 5\text{H}_2\text{O}$ ) in three holes bored into the trunk.

On July 11, samples were collected from each of the four trees. Samples consisted of (1) the top 6 inches of 1933 season's growth of topmost branches and leaves, (2) a similar portion of lower outside branches, and (3) entire 1933 season's growth of short inside shoots. The two trees healthy on June 22 were still so when sampled. Of the affected trees on that date, the treated one was making healthy growth; whereas leaves of the top branches in the untreated one were severely chlorotic and there was some necrosis of leaves and shoots. The samples collected of outer side branches were mildly chlorotic. The short inside shoots were healthy.

On July 14 a fifth mildly affected tree having a height of only 15 feet was treated as shown in figure 1, 10 g of zinc sulphate being used

for each application. On August 18 samples for analyses were collected from this tree as follows:

(1) New growth on girdled branch. Shoots here had made only 2 inches of growth since girdling. There were no symptoms of rosette and no union of cambial or cortical tissue at the girdle.

(2) New growth on defoliated branch. The defoliating had been done only once so that new growth had its full complement of leaves. Shoots had made 6 inches or more of growth since defoliation. No symptoms of rosette.

(3) New growth on an untreated side branch, check for 1 and 2. Samples displayed mild chlorosis of leaves, but shoots were not necrotic.

(4) New growth on treated branch in top of tree. No symptoms of rosette.

(5) New growth on untreated branch in top of tree. Leaves were mildly chlorotic, but no necrosis of leaves or shoots were found.

#### SEASON OF 1934

On October 16, 1934, samples were collected of what was thought might be wider differences of healthy and affected tissue than were represented in the samples of 1933. These consisted of leaves collected at the tips of 1934 season's growth in the top of 10 rosetted and 9 healthy trees in the Yuma Valley. The rosetted trees were 5 to 8 years of age and located in four orchards along the east side of the valley. Rosette has been common there. The trees sampled were known to have been rosetted for the preceding three summers. The healthy trees were from a single 8-year-old orchard on the west side of the valley where rosette has been unknown.

#### CHEMICAL ANALYSES

All samples were washed in distilled water as soon as collected and were subsequently dried in the sun or in the draft of an electric fan and stored in the laboratory until analyzed during the winter following collection. The samples were broken into small pieces, placed in a silica or platinum dish, dried overnight at 110° C., and weighed. This was the dry weight upon which all percentages were based. Leaf samples generally weighed from 90 to 100 g, and stem samples 20 to 40 g. Samples were ashed at dull redness in an electric muffle. The ash was weighed, then taken up with dilute hydrochloric acid. Analyses were made upon aliquots of this for the various elements using the methods of the Association of Official Agricultural Chemists (3).

This method for the determination of zinc consists of separating zinc from copper by precipitation of the copper with hydrogen sulphide in solution, containing 5 cc of concentrated hydrochloric acid in 250 cc, then by precipitating zinc as the sulphide with the solution adjusted to a pH of 2 to 3. The zinc is estimated turbidimetrically as the ferrocyanide. It is recognized that the method is not completely accurate. The micromethod described by Hibbard (13) is probably an improvement, and he provided a copy of his method before analyses were begun on the material collected in 1934. It was deemed best to continue with the official method since it had been used the two previous years. Furthermore, the use of large samples (18 to 20 times as large as those suggested by Hibbard) would tend to reduce errors incidental to analyses.

## DISCUSSION OF DATA

The results as found for healthy and rosetted pecan trees, untreated and treated by placing zinc sulphate in the trunk are shown in table 1 and figure 2. Similar data for some parts of the tree given special

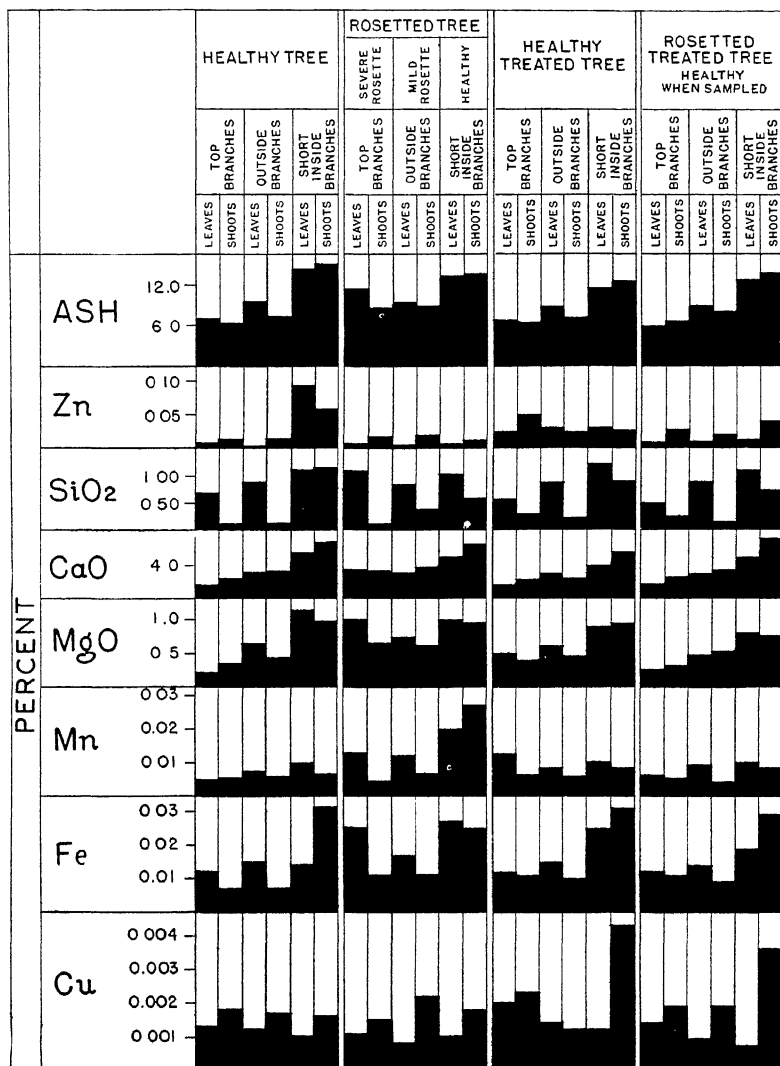


FIGURE 2.—Analytical data from several trees sampled in 1933. These trees were growing in a district where rosette has been common. The healthy, untreated tree was probably close to a rosetted condition when sampled, for mild symptoms appeared later in the summer.

treatments as shown in figure 1 are shown in table 2 and figure 3. The data for zinc and ash content of the healthy and diseased tissue collected in the Yuma Valley in 1934 are shown in table 3 and figure 4.

The zinc content was also calculated as the percentage of total ash in the tissue; this tended only to show wider differences in zinc content and the data computed in this way are not included.

TABLE 1.—Percentage of oven-dry weight, ash, and certain mineral constituents in tissues of healthy and rosetted trees when sampled July 11, 1933

Condition of tree	Branches sampled	Condition	Tissue	Ash	Silica	CaO	MgO	Mn	Fe	Cu	Zn
Healthy	Top	Healthy	Leaves	7.12	0.553	1.83	0.2306	0.0056	0.0129	0.0013	0.0088
			Shoots	6.42	.108	2.29	.3811	.0058	.0075	.0018	.0143
	Side	do	Leaves	9.72	.724	3.09	.6612	.0078	.0154	.0012	.0046
			Shoots	7.92	.114	3.13	.4476	.0062	.0077	.0017	.0149
Rosetted <sup>1</sup>	Short inside	do	Leaves	14.46	.916	5.45	1.1450	.0102	.0140	.0010	.0332
			Shoots	15.12	.930	6.77	.9906	.0067	.0319	.0016	.0375
	Top	Severe rosette	Leaves	11.54	.886	3.51	.0192	.0134	.0253	.0011	.0076
			Shoots	8.45	.105	3.49	.6644	.0048	.0110	.0015	.0187
Healthy, treated <sup>2</sup>	Side	Mild rosette	Leaves	9.29	.680	3.03	.7552	.0123	.0171	.0008	.0062
			Shoots	8.49	.319	3.73	.6367	.0061	.0113	.0022	.0191
	Short inside	Healthy	Leaves	13.16	.837	4.95	.0726	.0200	.0270	.0010	.0074
			Shoots	13.76	.475	6.39	.9763	.0272	.0250	.0018	.0125
Previously rosetted, treated <sup>3</sup>	Top	do	Leaves	6.50	.469	1.69	.5177	.0129	.0120	.0020	.0251
			Shoots	6.32	.245	2.19	.4174	.0063	.0112	.0023	.0506
	Side	do	Leaves	8.47	.717	2.91	.6223	.0084	.0158	.0014	.0318
			Shoots	6.88	.192	2.31	.4622	.0061	.0103	.0012	.0226
	Short inside	do	Leaves	11.64	.987	3.98	.9052	.0103	.0257	.0012	.0312
			Shoots	12.48	.723	5.44	.9517	.0088	.0310	.0043	.0279
	Top	do	Leaves	5.88	.403	1.70	.2734	.0068	.0129	.0014	.0074
			Shoots	6.74	.205	2.45	.3368	.0058	.0115	.0019	.0285
	Side	do	Leaves	8.75	.718	2.89	.4997	.0094	.0149	.0009	.0103
			Shoots	7.86	.128	3.28	.5420	.0044	.0095	.0019	.0199
	Short inside	do	Leaves	12.84	.887	4.90	.8163	.0102	.0193	.0007	.0111
			Shoots	13.82	.590	7.11	.7722	.0086	.0293	.0036	.0403

<sup>1</sup> Samples of the topmost leaves of this tree had severe chlorosis, the topmost shoots showed some necrosis, the outside branches had chlorotic leaves but no necrosis, and the inside branches were virtually free of rosette symptoms.

<sup>2</sup> This healthy tree was treated by placing 30 g of commercial zinc sulphate ( $\text{ZnSO}_4 \cdot 5\text{H}_2\text{O}$ ) in its trunk June 22, 1933.

<sup>3</sup> This tree was rosetted prior to June 22 when it was treated as was the healthy tree (see note 2), it was healthy when sampled.

TABLE 2.—Percentage ash and certain mineral constituents in tissues from the tree shown in fig. 1 which was treated on July 11, 1933

[Samples for analysis collected Aug 18, 1933]

Position of branches	Treatment	Condition	Tissue	Ash	Silica	CaO	MgO	Mn	Fe	Cu	Zn
Top	Untreated	Mild rosette	Leaves	6.66	0.527	1.96	0.5350	0.0088	0.0165	0.0010	0.0068
			Shoots	6.52	.237	2.41	.3877	.0042	.0156	.0016	.0202
			Average	6.59	.382	2.18	.4613	.0065	.0175	.0013	.0135
	Treated	Healthy	Leaves	5.21	.425	1.33	.3946	.0058	.0161	.0009	.0849
			Shoots	5.47	.261	1.69	.3048	.0043	.0153	.0009	.0749
			Average	5.34	.343	1.51	.3497	.0050	.0157	.0009	.0799
Side	Untreated	Mild rosette	Leaves	7.05	.476	2.06	.5813	.0081	.0207	.0008	.0074
			Shoots	7.85	.372	2.94	.5587	.0068	.0216	.0016	.0207
			Average	7.45	.424	2.50	.5700	.0075	.0211	.0012	.0140
	Treated and girdled	Healthy	Leaves	8.51	.789	2.59	.6926	.0119	.0248	.0008	.0258
			Shoots	7.88	.241	3.11	.5127	.0041	.0148	.0010	.0478
			Average	8.20	.515	2.85	.6026	.0080	.0198	.0009	.0368
	Treated and defoliated	do	Leaves	5.72	.647	1.37	.4895	.0059	.0203	.0011	.0361
			Shoots	6.79	.712	1.92	.4220	.0043	.0028	.0020	.0435
			Average	6.25	.679	1.65	.4557	.0051	.0115	.0015	.0398

## ASH AND MINERAL CONTENT OTHER THAN ZINC

The data throw light upon the mineral content of healthy and rosetted pecan trees, especially with regard to the consistent difference in ash content between different types of shoots or parts of the tree

PERCENT		TOP BRANCHES				OUTSIDE BRANCHES					
		UNTREATED ROSETTED		TREATED HEALTHY		UNTREATED ROSETTED		TREATED AND GIRDLED HEALTHY		TREATED AND DEFOLIATED HEALTHY	
		LEAVES	SHOOTS	LEAVES	SHOOTS	LEAVES	SHOOTS	LEAVES	SHOOTS	LEAVES	SHOOTS
ASH	5.0										
	Zn 0.05										
	SiO <sub>2</sub> 0.5										
	Ca O 3.0 2.0 1.0										
	Mg O 0.5										
	Mn 0.01										
	Fe 0.02 0.01										
	Cu 0.002 0.001										

FIGURE 3 —Results of analysis of the tree given special treatment as shown in figure 1.

(table 1 and fig. 2). Short inside branches in the lower part of the tree had consistently a greater ash content than had the long growths in the top of the tree. The outside shoots, which are intermediate in length and vegetativeness, were generally intermediate in percentage of ash.



Along with the difference in mineral content of branches from different parts of the tree, there appears to be a relation between development of rosette and ash content. The top branches, the most severely affected part of the rosetted tree, had a much higher ash content than had comparable samples from healthy trees (table 1 and fig. 2). In the tree given special treatment (table 2 and fig. 3) the treatment of one top branch with zinc (fig. 1) and the subsequent production of healthy growth was accompanied by a reduced ash content. The data of 1934 (table 3 and fig. 4) are also indicative of a higher ash content in rosetted than in comparable healthy tissue. Haas (12) has reported a higher ash content in rosetted than in healthy walnut and pecan tissue.

However, in the rosetted tree (table 1 and fig. 2) the short inside shoots which were entirely free of rosette symptoms were higher in ash than were the rosetted top shoots. It seems that the ash content is influenced by the condition of growth and location in the tree. The increased ash content found for rosetted tissue may be the result of rosette and not present in a causal relation. It is only when growths of similar type or vegetativeness are considered that the relation between ash content and rosette symptoms is apparent.

Further evidence that the mineral content is influenced by the amount or quality of the growth as well as the health of the tissue is seen in the results following girdling and defoliation (table 2 and fig. 3). The growth produced after girdling had a somewhat increased ash content; that produced after defoliation had a reduced ash content. Following girdling, the shoots were short and otherwise resembled those on the inside of the tree. Following defoliation, the growth was longer, slender, and willowy, approaching the character of the healthy topmost shoots where the ash content was low. Samples were of growth following defoliation and girdling and both were of the same age.

TABLE 3.—Percentage of ash and zinc content of leaves at top of rosetted and healthy trees of several varieties in the Yuma Valley

[Collected Oct 16, 1934]<sup>1</sup>

Variety	Condition	Ash	Zinc
Kincaid.....	Rosetted.....	8.96	0.003
		8.01	.0071
		7.73	(2)
		7.36	(2)
	Healthy.....	7.37	.0202
		6.11	.0136
Halbert.....	Rosetted.....	6.24	.0153
		8.06	.0053
		7.84	(2)
		7.63	(2)
	Healthy.....	5.07	.0157
		5.54	.0174
Success.....	Rosetted.....	5.34	.0066
		7.71	.0048
		8.50	(2)
		7.43	(2)
	Healthy.....	6.59	.0163
		6.03	.0145
		6.38	.0084

<sup>1</sup> The rosetted trees were on the east side of the valley where rosette is somewhat common. The healthy trees were on the west side of the valley where it is unknown.

<sup>2</sup> Traces.

What was true of ash content has been generally true for each of the elements determined except zinc. A comparison of the analyses of the topmost tissue in the healthy untreated and in the rosetted tree indicates that silica, calcium, magnesium, manganese, and iron may be higher in rosetted than in comparable healthy tissue. At the same time, the rosetted top shoots were for the most part no higher in these materials than were the healthy, short inside shoots. For the rosetted tree alone, the latter were highest in the various elements. There is a general trend in the data from all of the healthy trees for the short, inside shoots to be highest in all elements and for the outside shoots to be intermediate. Similarly, in the untreated, girdled, and defoliated outside shoots, the calcium, magnesium, manganese, and iron were reduced in the defoliated tissue and were high or increased in the girdled or untreated tissue. Copper appears to bear little relation to condition of health or type of growth.

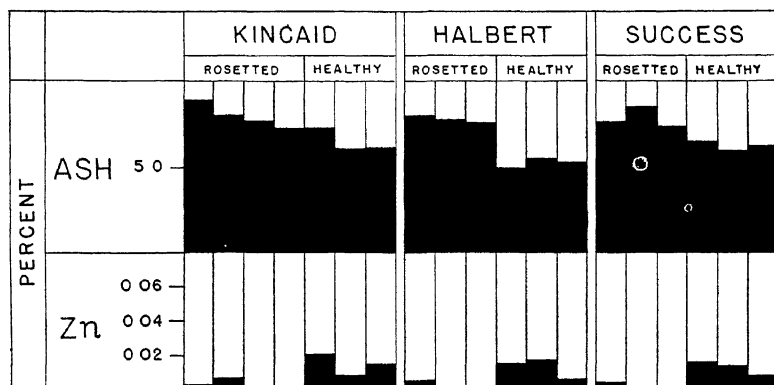


FIGURE 4.—Ash and zinc content in topmost leaves of rosetted and healthy pecan trees in the Yuma Valley, collected October 15, 1934. These trees represented what are believed to be wider differences between a healthy and rosetted condition than those of figure 2.

#### ZINC CONTENT

The zinc content found in the various tissues exhibits somewhat more of a specific relationship to the presence or absence of rosette symptoms than do the other elements determined. This is particularly true of the samples in table 3 and figure 4. These samples, collected from comparable parts of healthy and rosetted trees, indicated a higher zinc content in the healthy than in the diseased tissue. These healthy samples were from an area in which rosette has never been known, whereas the affected ones were from an area where rosette has been common. Greater differences in fundamental factors responsible for rosette might be expected than in the samples (table 1 and fig. 2) which were all from a locality where rosette has been serious.

In the analysis of 1933 (table 1 and fig. 2) the zinc content found for the untreated rosetted and healthy trees was significantly different only in the short inside shoots where it was markedly less in the rosetted than in the healthy tree. The top branches of these two, which differed widely in degree of health, and the outside branches, which were mildly affected in the rosetted tree and unaffected in the

healthy tree, contained practically the same percentage of zinc. Though the total zinc content was not greatly different, the proportion of zinc to total ash was much reduced in the rosetted tissue.

Data from the rosetted tree alone (table 1) presents an analogous condition. The topmost part which displayed the most extreme symptoms of rosette contained virtually the same percentage of zinc as did the healthy, short, inside shoots and the less severely affected outside shoots.

Following the application of zinc the treated rosetted tree produced healthy growth in the top and outside branches. This was accompanied by but a very slight increase in total zinc content over that of the untreated tree (table 1 and fig. 2). The proportion of zinc in the ash was greatly increased. It would appear that if pecan rosette is related to quantitative amounts of zinc in the tissue, the differential between healthy and affected tissue is small. There is also the possibility that when small amounts of zinc are present, its action is modified by the presence of other minerals occurring in the ash. Chandler, Hoagland, and Hibbard (5) have reported some overlapping of zinc content between healthy and little-leaf tissue, generally more zinc being found in the healthy tissue.

In the healthy, untreated tree (table 1) where zinc might be expected to have a normal distribution within the tree, the short inside shoots contained markedly more zinc than the top or side branches. These shoots were from the lower part of the tree. Such shoots are not commonly affected with rosette, even in trees where the topmost parts are badly diseased. While the movement of zinc following treatment is most rapid to the top and rapidly growing parts, its normal accumulation seems to be greatest in the lower, slowly growing branches. Perhaps this is why they are less susceptible to rosette. However, other conditions are not equal, for the upper part of the tree is more exposed to light and heat.

Different parts of trees all equally healthy in appearance have been found to contain widely different amounts of zinc. Apparently after a minimum requirement is satisfied, additional quantities of zinc serve no further need. The appearance of healthy trees was not affected by the addition of zinc.

The absence of a quantitative relation between amount of growth and zinc may indicate that it does not enter extensively into the protoplasmic structure. Reed and Dufrenoy (19) suggest that it may act in regulating the oxidation-reduction potential within the cells of citrus leaves. They believe that for an appreciable amount of oxidation, "the formation of complexes with metals which readily change their valences, thereby serving for H and O transfers" is necessary. "Two or more molecules of amino acids may be linked with one of a metal such as iron, copper, manganese, or zinc."

That a metal which readily undergoes a change in valence could be important physiologically as an oxygen acceptor and thereby function in maintaining the oxidation-reduction potential of the cell is a tenable theory. However, zinc is not such a metal. According to Mellor (16) zinc belongs to a family of elements (group 11 in periodic arrangement) of which only mercury readily undergoes a change in valence. Even here the lower oxide is unstable. Zinc

forms the monoxide after the formula:  $2\text{Zn} + \text{O}_2 = 2\text{ZnO}$ . In this the valence is typically 2. The only other oxide of zinc is the peroxide having the chemical structure:



In the peroxide the valence of zinc is unchanged. In forming this compound, oxygen rather than zinc undergoes a change in valence. Zinc being bivalent could act as a nucleating element joining two molecules of amino acids or similar groups, as Reed and Dufrenoy point out, but judged by the known properties of zinc, there could be no auto-oxidation of the amino acids resulting from valence changes at the union. Such a function for zinc cannot be postulated, as it might well be for iron, copper, or manganese.

Zinc has been reported by several workers to be necessary for the growth of green plants. It has also been known for many years to be beneficial to the growth of fungi. The sum total of the knowledge of zinc in plants is yet small. The present data do not indicate that any large amount of zinc is held in affected tissue unavailable for physiological processes. Rather, they would tend to suggest that rosette symptoms are produced when the total zinc in the tissue is insufficient for normal metabolism. Data previously presented (8) support this view as do those of Chandler, Hoagland, and Hibbard (5) and Reed and Dufrenoy (19) for other horticultural plants. Also, substantiating this interpretation is the fact that in earlier studies (8), acids injected into affected trees did not bring about recovery through making available any elements or ions already present. Similarly, the application of sulphuric acid to the soil around affected trees in a district where rosette is serious was not effective. It has previously been reported that no relation between soil alkali or salt content and rosette occurred in Arizona (8). The occurrence of pecan rosette on the acid soils of the South has been reported by Skinner and Demaree (20) and others. Studies of the hydrogen-ion concentration of healthy and rosetted leaves and stems have not revealed consistent differences in reactions.

It seems evident that if any portion of zinc in the tissue is not available for metabolism, it is a much smaller portion than is the case with some other elements. Iron, for example, may be more abundant in tissue, chlorotic for lack of utilizable iron, than in healthy tissue. The greater solubility of zinc may account for its being more readily available. The ultimate success of controlling pecan rosette through treatment with zinc will depend considerably upon whether only supplying a deficiency is necessary or whether measures to influence its availability in the tree are also involved.

This matter may also have bearing upon the fluctuation and irregularity of occurrence of rosette. If zinc is not held unavailable in appreciable quantities in the tree, then the development of the disease on healthy trees would point to soil fixation. It has been frequently observed in Arizona that where corrals or chicken pens have been built around trees, rosette soon follows. Chandler et al. (5) have reported that corral soils have a high zinc-fixing power.

## MOVEMENT OF ZINC IN THE TREE

The treatments given the tree shown in figure 1 were designed especially to give information upon the movement of zinc in the tree. This was deemed important especially in view of the erratic occurrence of rosette wherein one limb of a tree may be affected and the rest healthy, and wherein the top of the tree is almost always more severely affected than the lower parts.

The samples collected from the top branches 35 days after one branch had been treated with zinc revealed wide differences in zinc content (table 2 and fig. 3). The average zinc content in the tip of the treated branch was approximately six times that in the untreated. In the latter, the content was not increased above that for comparable tissue in the untreated trees (table 1). Since it is impossible to know the zinc content of the same tissue both at time of treating and subsequent thereto, it cannot be known if a small increase occurred in the untreated branch. There had been some slight improvement in the health of the untreated branch, and it is possible that the zinc content had increased slightly. It is evident, however, that the greatest movement of zinc was upward in the treated branch and that little moved downward to the junction with the untreated branch and thence upward in it.

The zinc content found in the lower branches supports this interpretation. The treatment in one lower branch (table 2 and figs. 1 and 3) did not increase the amount of zinc found in the outside shoots of the untreated branch on the opposite side of the tree, beyond that found for the outside branches of the untreated trees (table 1). Here again, severe rosette symptoms had not developed, nor had they developed anywhere in the tree. This fact is taken as indicating some migration of zinc downward from the point of treatment to the trunk where it joined the general vascular supply of the tree. If such a migration downward occurred, the amount was not great enough to be measured. Clearly, the most immediate and abundant movement of zinc is in an acropetal direction. In the field treatment of many trees, it has been observed that limbs above the treatment are the first to respond, but the entire tree eventually does so.

The severely rosetted condition of one branch of an otherwise healthy tree may be accounted for by the tendency of zinc to move upward in the tree. In such cases, one might suspect the absorption of zinc by the roots below the affected limb.

The healthy tree treated with zinc in 1933 revealed an increased zinc content over the healthy untreated tree only in the top and outside branches. The increase was greater in the side branches. There was no increase in the short inside branches. This might be interpreted as chemical evidence of a fact many times observed in the treatment of rosetted trees with zinc; the topmost shoots, while most severely affected are first to recover, and rapidly growing shoots respond more quickly than slowly growing ones. In young, vigorously growing trees the response of zinc applied to the trunk has often been noted on young leaves in 3 days following treatment. Zinc applied in excess amounts has produced a toxic effect on the leaves in 24 hours.

Girdling of a limb on the low branch (fig. 1) did not prevent the passage of zinc from the point of treatment upward, for the sample

from above the girdle contained markedly more zinc than did the outside branches of any of the trees sampled. The movement of zinc upward must then be in the xylem. The new shoots on the defoliated limb likewise were found to have an abnormally high zinc content. Seemingly, the reduction in leaf area adjacent to the new growths did not affect the movement of zinc into them.

#### SUMMARY

The present paper reports additional evidence that the occurrence of pecan rosette is to a considerable extent associated with an insufficiency of zinc for normal metabolism in the tissue. Such factors as condition of growth, location in the tree, exposure to light and heat, and probably many others may act with a reduced zinc content to cause the development of rosette symptoms. No evidence was found that any appreciable quantity of zinc is present but not utilizable in affected tissue.

The total ash content was found to bear a relation to the occurrence of rosette when shoots of similar growth character and position in the tree were compared. In comparisons of this kind, the ash of affected tissue was found to be higher than that of healthy tissue. Since other factors influence the ash content so markedly, we cannot look upon it as occurring in a causal relation to pecan rosette. It is more probably a result.

The translocation of zinc occurred in the xylem. Zinc, when placed directly in the xylem, moved most rapidly in an acropetal direction. A slower lateral and downward migration probably occurred.

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# THE ISOLATION OF A HEMOPHILIC BACILLUS IN PURE CULTURE AND THE REACTION OF CHICKENS TO EXTRANASAL INOCULATIONS THEREOF<sup>1</sup>

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## INTRODUCTION

The study of hemophilic bacilli herein reported deals with two different phases: (1) A technique of isolating *Hemophilus gallinarum* in pure culture; and (2) the reaction of chickens to the extranasal inoculations thereof.

## ISOLATION OF HEMOPHILIC BACILLI IN PURE CULTURE

In a previous publication (2),<sup>2</sup> the writers reported their success in isolating hemophilic bacilli from the edematous facial swellings of chickens showing an infectious coryza. The technique consisted in employing several loopfuls of the edematous fluid from the swellings to inoculate a blood medium. After 24 hours incubation at 37° C. the microscopic examination of the medium usually showed a pure growth of these organisms.

Since the time of that report, an opportunity of studying infectious material from other than the source used in the previous work has become available, and the technique has been found applicable to that type of infection with which edematous swellings about the face are commonly observed.

Certain difficulties were experienced in isolating the organisms directly from the edematous facial swellings of fowls from field outbreaks, but susceptible chickens, infected under laboratory conditions with material from these field cases and examined within 24 hours after swellings were noted, yielded pure cultures from each of the various field sources of the disease. It is apparent that the duration of the infection in the individual fowls from the field outbreaks was unknown and since, in the previous work of the writers, the cultures had been made when the swellings were first noted, the time factor seemed to be of significant importance in obtaining positive results.

De Blicke (1), Nelson (4, 5, 6), Schalm and Beach (7), and Eliot and Lewis (3), have described their method of isolating the organisms from the nasal exudate. They have also shown the significance of these organisms in producing a respiratory infection in susceptible fowls. In the writers' experience, the isolation of the organisms from the nasal exudate is somewhat tedious and more difficult to accomplish than is isolation from the swellings, although it probably constitutes the only manner in which they may be isolated in those cases showing only a nasal exudation.

<sup>1</sup> Received for publication Oct. 1, 1935; issued April, 1936. Published as Contribution No. 474 of the Rhode Island Agricultural Experiment Station.

<sup>2</sup> Reference is made by number (italic) to Literature Cited, p. 383.



## MATERIAL AND METHODS

## PREPARATION OF MEDIA

The blood was obtained directly from the heart of chloroformed fowls after the keel and ribs had been cut in such a manner as to expose the heart and was collected in a sterile glass hypodermic syringe and placed in a sterile 2-percent sodium citrate normal salt solution at the rate of 5 cc of blood to from 10 to 20 cc of this solution. In this manner from 15 to 20 cc or more of sterile whole blood could be obtained from the average fowl.

When media were desired for use, the citrated blood was added to nutrient agar slants so that it extended from three-fourths to 1 inch from the bottom of the slant. It was found desirable to use fresh blood and make up the media just before they were used.

## METHOD OF ISOLATION

The skin over the edematous swellings of the fowls to be examined was seared, and an incision through the seared skin was made with a hot instrument. Two to three loopfuls of the edematous fluid were used to inoculate each of several tubes of the media as described above.

After these had been incubated at 37° C. for 24 hours, smears were made from each culture tube and stained with Gram's stain. The microscopic examination of stained smears constitutes the only sure way of detecting growth as the changes occurring from growth in this medium are only slight except for the coagulation of the blood portion which may, at times, occur within this period.

A modification of Gram's method of staining may be used to good advantage. It consists in substituting gentian violet made as follows: A, 4 g of gentian violet in 25 cc of 95-percent alcohol; B, 14 cc of aniline oil and 2.5 cc of hydrochloric acid in 500 cc of distilled water. The iodine solution is made up of a 1-1,000 solution of tincture of iodine in water. This stain is used in the usual manner except that the safranin is allowed to stain from 1 to 2 minutes or longer. With this stain the smear has a pink background, and the bacteria stain a more intensive pink.

CULTURAL AND MORPHOLOGICAL CHARACTERISTICS OF *HEMOPHILUS GALLINARUM*

The morphological and cultural characteristics of *Hemophilus gallinarum* have been described by the writers in a previous publication (2, pp. 7-8). The organisms colonize on blood-agar slants and plates if grown in an atmosphere containing sufficient moisture (2), or in an atmosphere containing 10 percent of carbon dioxide as reported by Schalm and Beach (7).

Stained preparations from blood-agar slants or plates usually show the organisms to be larger than those grown in the blood-citrate-salt solution at the base of nutrient agar slants.

Thus far, attempts to grow the organisms on other than a blood medium have not been satisfactory, although growth in a medium consisting of small pieces of sterile raw potato in broth and sodium citrate solutions at the base of nutrient agar slants have shown scanty growth as indicated by stained preparation of the media.

Pure culture of the organisms grown in blood at the base of nutrient agar slants produce swellings when inoculated subcutaneously in chickens and also cause a systemic reaction as indicated by the birds appearing depressed.

Stock cultures maintained in the blood at the base of nutrient agar slants have been found capable of inducing respiratory symptoms when inoculated into chickens after a period of 10 months although the virulence is greatly reduced at this time.

#### EXPERIMENTAL DATA

Bacteriological examinations were made of the edematous material from facial swellings of 10 field cases from 5 sources. *Hemophilus gallinarum* was isolated from only one bird representing a field case of the infection.

Eight of nine susceptible fowls inoculated from the respective field infections gave positive results when the material from their edematous swellings was used to inoculate the same type of medium within 24 hours after the swellings were first noted. The other fowl was not examined.

As nothing was known with respect to the length of time the infection had persisted in any of the individual field cases, this factor seemed to be of considerable significance in the successful isolation of the organisms. Tests were therefore conducted with a series of adult Barred Plymouth Rock and White Leghorn chickens which were inoculated intranasally to determine the practicability of isolating organisms from fowls within 24 hours after the swellings about the face were noted, and also after 48 hours.

The bacillus was isolated from five of the six birds within the 24-hour period, whereas the results were negative in five of the six fowls examined at the 48-hour period.

The results would indicate that the bacilli of *Hemophilus gallinarum* are fewer in numbers or have ceased to grow at some period 24 hours after the swellings have developed. This probably runs parallel to the course of the infection, for on an average the swellings ordinarily persist for only a few days and recovery is usually apparent within a week or 10 days from the onset of the infection in the particular strains studied.

#### THE REACTION OF CHICKENS TO THE EXTRANASAL INOCULATIONS OF HEMOPHILIC BACILLI

The ability of certain hemophilic bacilli to induce an inflammation of the upper respiratory tract of chickens has been reported by De Blieck (1) Nelson (4, 5, 6), Delaplane, Erwin, and Stuart (2), Schalm and Beach (7), and Eliot and Lewis (3). A certain degree of resistance to reinfection has been noted in chickens that have recovered from the field type of infection. These chickens are particularly resistant to the intranasal inoculations of cultured organisms, while those recovering from culturally induced infection are quite resistant to reinfection with the same cultured organisms but are not completely protected against the disease as found in the field.

Extranasal inoculation of cultured organisms was made to note the reaction in chickens and to determine whether such a procedure would induce resistance to intranasal infection with the same strain of organisms.

In a preliminary small group of chickens, it was noted that the subcutaneous inoculation of freshly isolated cultured organisms was followed by swellings 24 hours after inoculation. These birds were quite lame and considerably depressed for a few days but subsequently recovered and were found resistant to intranasal inoculations of the cultured organisms and the field infection 2 weeks after the subcutaneous injections had been made.

#### MATERIAL AND METHODS

The chickens constituting the various experimental groups were of different breeds and ages at the time of use but each group of birds was of the same breed and age. They were reared on the poultry farm at the experiment station and were known to have been free of clinical evidences of respiratory infections prior to experimentation.

The culture material used for the various groups was 24-hour cultures grown in a blood citrate solution and was diluted just prior to use with sterile normal salt solution.

The subcutaneous inoculations were made in the skin over the leg, a 20-cc syringe and an 18-gage needle being used. The skin area at the point of injection was painted with tincture of iodine before and after the inoculations were made. The technique for the intraperitoneal and wattle inoculations was similar to that used for subcutaneous inoculations in that the point of inoculation was prepared in a similar manner.

#### EXPERIMENTAL DATA

The primary tests were conducted on a group of adult Rhode Island Red pullets. Six birds received the cultural organisms subcutaneously in the skin area over the leg, and three were injected in the wattles. The two groups were maintained in separate quarters. Twenty-four hours following the inoculations, swellings were evident at the point of inoculation of both groups. The six birds receiving the subcutaneous injections were considerably depressed and lame for a few days but gradually became normal. Two weeks later they were inoculated intranasally with material from a transfer of the same culture used for the injections. They were resistant to this intranasal inoculation. Still later these same birds were inoculated with the nasal exudate from fowls which had been infected with the exudate of the field form of the disease, and from which the organisms had been originally isolated. They were found resistant to this field form of the disease. The control chickens obtained from the same source as the ones used for the tests were susceptible to the cultured organisms and the field infection. The three birds that had been injected in the wattles contracted intranasal infection within 72 hours after the injection and were not tested after they recovered.

The results of the preliminary tests were so encouraging that it was planned to conduct the experiment on a larger scale. Two groups of White Leghorn pullets consisting of 40 birds each comprised the initial groups. The two groups were maintained in separate pens and each received 0.5 cc of a 50 percent 24-hour culture solution subcutaneously over the right leg. One group received the organisms from culture 1,<sup>3</sup> and the other from culture 2.<sup>4</sup> They were quite

<sup>3</sup> Culture isolated from the Rhode Island type of this disease.

<sup>4</sup> Cultures obtained from other than Rhode Island sources

depressed over a period of 5 days after the inoculations had been made, and a number were lame in the leg receiving the inoculation.

Twenty days following the subcutaneous inoculations a few of the birds of each group which had not been inoculated intranasally or been in contact with cases of the disease, contracted intranasal infection. Following the observation of the first few cases, the remainder of each group developed a coryza.

The effect of the intranasal inoculation of 19 of the fowls which had been inoculated with culture 1 was tried after they had recovered from the subcutaneous inoculations and the subsequent intranasal infection which they had contracted. All the birds except one were found resistant to reinfection from cultures 1 and 2.

The intranasal reinoculations of eight of the birds originally inoculated with culture 2 were made after they had recovered from the subcutaneous inoculations and the subsequent intranasal infection. They were all resistant to inoculations of cultures 1 and 2.

Ten 4-week-old Barred Plymouth Rock chickens were used in a study of the effect of the subcutaneous inoculation. These chicks were confined in a battery brooder in isolated quarters, and special precautions were taken to be sure that any leakage from around the needle at the time of inoculation was promptly sponged with cotton saturated with tincture of iodine. These precautions were taken to avoid accidental intranasal infection from extraneous material.

These birds received two injections of culture 1, the second being made 10 days after the first. Each of the chickens showed swellings at the point of inoculation 24 hours following each injection. They were somewhat depressed and lame for a period of 5 days following the first inoculation but did not appear to have reacted so severely as the adult birds. These chicks contracted intranasal infection 23 to 25 days after the first inoculation in spite of the precautions taken to make sure that any material leaking from around the needle could be at fault, thus indicating that intranasal infection might occur from the subcutaneous inoculation of cultured material.

The reaction of the 10 birds to intranasal reinoculation was also tested after they had recovered from the intranasal infection. Two of the chickens contracted intranasal infection as a result of the reinoculation with cultured organisms of culture 1.

Five Rhode Island Red pullets were injected intraperitoneally with cultured material on October 29 and November 1 and were maintained in adjoining separate cages of a laying battery. These birds did not show any appreciable reaction to the intraperitoneal injections. Four of them contracted intranasal infection 9 days after the first inoculation.

It was considered of interest to determine whether the combining of cultured organisms with laryngotracheitis virus and inoculating the material in the cloaca would offer any protection. Five 6-week-old Barred Plymouth Rock chickens were inoculated in the cloaca with this material, culture 39 being used with the laryngotracheitis vaccine. Four days after the inoculation of the combined material, all chicks showed extensive cloacal reactions. None of the chicks contracted laryngotracheitis or intranasal infection as a result of the inoculation. Nine days later they were inoculated in the larynx with laryngotracheitis vaccine and culture 39 and were found resistant.

Five days later they were inoculated intranasally with cultured organisms, and after 24 hours they all showed intranasal infection, showing that no resistance had developed from the cloacal injections 2 weeks after the inoculation.

#### SUMMARY AND CONCLUSIONS

Evidence is presented to show that *Hemophilus gallinarum* may be readily isolated in pure culture from the edematous swellings of fowls showing an infectious coryza and that the success of the isolation depends on the length of time the swellings have persisted. Thus, if the material is cultured within 24 hours after the swellings have developed, the isolation of the organisms is readily accomplished. After 48 hours, there is less chance of obtaining the organism. As the duration of time the infection has persisted in field cases is usually unknown, it is suggested that for diagnostic purposes susceptible fowls be inoculated intranasally with the infective material and cultures made from them within 24 hours after the facial swellings are noted.

The technique serves as an aid in the differentiation of certain other respiratory infections of fowls in which a coryza is a symptom, particularly that type characterized by a marked rattled breathing and lesions of an acute bronchitis and tracheitis with which a coryza is noted and which may also show some swelling around the eye. This particular infection is perhaps the same as that described by Beaudette and Hudson<sup>5</sup> at the Northeastern States Pullorum Disease Research Workers Conference. The infective agent of this disease readily passes through Berkfeld V and N filters as indicated by the ability of these filtrates to produce the typical disease condition in susceptible fowls. In contrast the filtrate of infectious coryza exudate does not produce infection when inoculated into susceptible fowls.

The extranasal inoculation of chickens with cultures of hemophilic bacilli is attended with reactions at the points of inoculation when given subcutaneously or in the wattle. The birds also show a systemic reaction as evidenced by those appearing depressed. The intraperitoneal inoculation of a small group of birds did not seem to result in any appreciable difference in their general health.

Intranasal infection occurred as a result of the subcutaneous injection of the cultured organisms. It would be difficult, however, to state the percentage that contracted the infection in this manner, since each of the birds of an experimental group was in contact with the others and when one developed intranasal infection, it was easy for the others to become infected from it.

No particular resistance to intranasal infection was noted in the birds receiving subcutaneous inoculations of cultured organisms, although approximately 3 weeks elapsed from the time of inoculation until they contracted intranasal infection.

The intranasal reinoculation of the birds recovering from the subcutaneous inoculations and the subsequent intranasal infection showed that all but one bird of the Leghorn-pullet groups and two from the Barred Plymouth Rock group to be resistant to reinfection.

<sup>5</sup> Unpublished data

Injection of the cultured organisms in the wattle was followed by intranasal infection. Four of five birds receiving intraperitoneal injections likewise contracted intranasal infection within 9 days after receiving the first inoculation.

The cloacal inoculation of the cultured organisms combined with laryngotracheitis virus did not result in any upper respiratory infection in a group of five chickens. The chickens were later found to be resistant to laryngotracheitis virus, but susceptible to intranasal inoculations of cultured organisms.

The fact that the preliminary group was resistant to intranasal reinoculations remains unexplained.

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# CHROMOSOME BEHAVIOR IN BLACKBERRY-RASPBERRY HYBRIDS <sup>1</sup>

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## INTRODUCTION

The genus *Rubus* in the United States is characterized by inter-specific compatibility, frequent lack of complete fertility, and a high degree of heterozygosity. This is shown by the taxonomic work of Brainerd and Peitersen (2)<sup>3</sup> on the New England blackberries, by the genetic studies on the same material by Peitersen (10), and by several investigations of Darrow and Longley (4) and of Longley (5, 6). This is further emphasized by the dewberry × raspberry cross made by Ness (9) in 1912. Both first and second generations

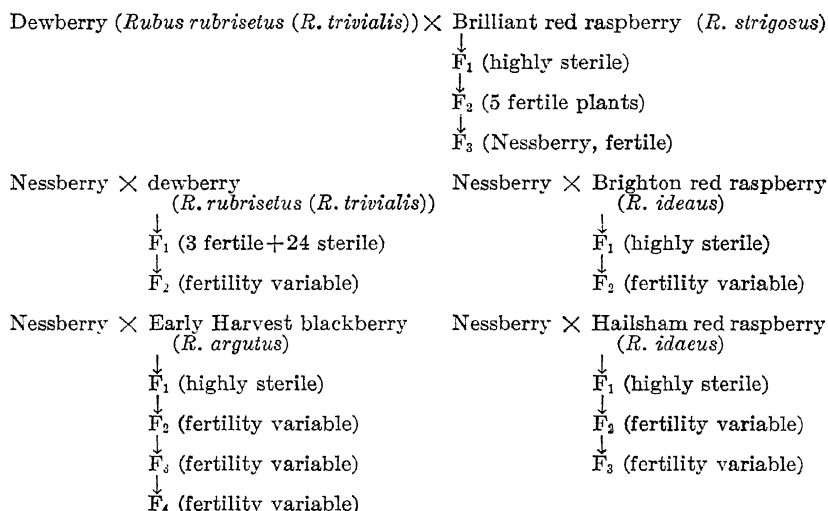


FIGURE 1—Diagrammatic representation of the origin and later crosses of the Nessberry with blackberry, dewberry, and raspberry.

of this cross (*Rubus trivialis* Michx. = *R. rubrisetus* Rydb. × Brilliant red raspberry, *R. strigosus* Michx.) were quite sterile with the exception of five F<sub>2</sub> (open-pollinated) plants, which were almost completely fertile, giving a third generation of 859 plants. Selections from these were given the name Nessberry. Ness then crossed this fertile hybrid to the dewberry parent, to two raspberries, and a blackberry and grew later generations. The last generation of each cross with the Nessberry, outlined in figure 1, has been available for cytological study.

<sup>1</sup> Received for publication Aug. 26, 1935; issued April, 1936.

<sup>2</sup> The writer acknowledges the assistance of Fraser Cowart, Dr. U. R. Gore, and Hennie Levy in the preparation of cytological material at different times.

<sup>3</sup> Reference is made by number (italic) to Literature Cited, p. 396.



The work here reported was undertaken to obtain further knowledge of chromosome behavior in the genus *Rubus*.

Since all of the crosses were made by the late Helge Ness, with whom the writer had no opportunity to discuss the material, portions of his notebook entries will be given in certain instances. An account of much of this work has been published by Ness (8, 9). In referring to his publications it must be remembered that he named the pollen parent first. For example, his paragraph heading (La  $\times$  (Brilliant  $\times$  La) F<sub>3</sub>) F<sub>1</sub> thus becomes the first generation of the back-cross Nessberry  $\times$  *Rubus rubrisetus* (*R. trivialis*) (9).

#### THE WILD DEWBERRY

Ness (9) considered *Rubus trivialis*, the dewberry found locally, and *R. rubrisetus*, secured for his work from Louisiana, as distinct species, based upon differences in shoot growth. It is doubtful, in view of the amount of variation in this respect observed in dewberries of this locality, if there is sufficient genetic difference between the two to justify this distinction. Root tips of the former have 14 chromosomes at metaphase (fig. 2, A). There are seven pairs at diakinesis, frequently joined by a single terminal chiasma. The movement toward the poles at the anaphase of the first division sometimes tends to be a little uneven (fig. 2, B), otherwise the division appears normal.

#### THE NESSBERRY

Longley and Darrow (7) illustrate the heterotypic division of the Nessberry with three figures. Diakinesis and metaphase exhibit 14 pairs of chromosomes: 1 pair appears to be lagging at telophase. They say: "It was exceptional to find irregularities during meiosis, but the history of this hybrid shows that it has been selected through several generations and now behaves like a stable species." A slightly greater degree of irregularity than the foregoing account would indicate has been observed in plants grown in Texas. Lagging has been noted during the heterotypic division, and in addition the chromosomes seem to have some difficulty in forming a normal metaphase plate. This interferes with an even movement toward the poles during the anaphase of the first division (fig. 2, C).

#### NESSBERRY $\times$ RUBUS RUBRISETUS F<sub>2</sub>

The first generation of the cross Nessberry  $\times$  *Rubus rubrisetus* is discussed by Ness (9, pp. 22-23). The cross was described before it was known that the Nessberry has twice the chromosome number of its parents. This first generation consisted of 27 plants—

intermediate of the parents, except three individuals which show only the Nessberry in all parts and bear a heavy crop of perfect fruits; all the others are more or less sterile, with canes showing traces of both parents. \* \* \* The leaves have the form and size of the Nessberry but their texture and surface are characteristic of the blackberries. The flowering is abundant, but each resulting fruit consists of a few scattered drupelets.

The simplest explanation of these results would seem to be that 3 tetraploid plants of the maternal type and 24 triploid true hybrids had been obtained. None of this generation has been available for study.

A second generation was grown on two occasions. Seed was sown on October 15, 1924, from F<sub>1</sub> plants of the Nessberry type. Five of

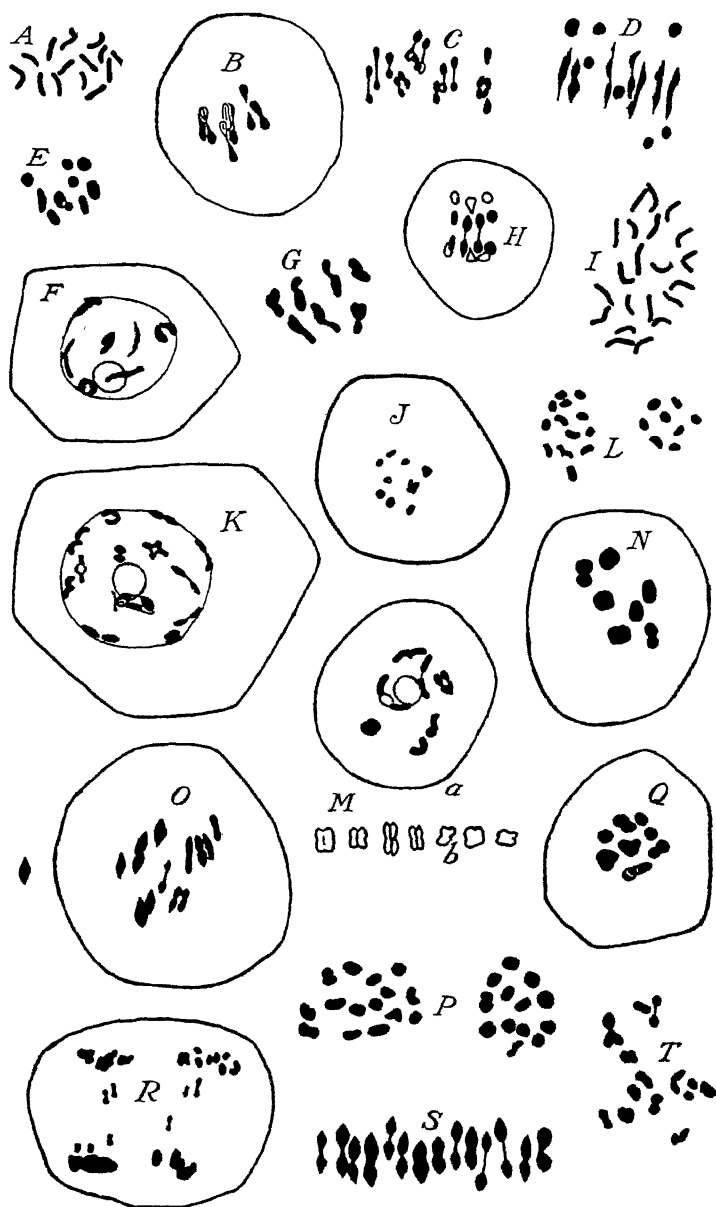


FIGURE 2.—1. *Rubus trivialis*, root tip. B, *R. trivialis*, first anaphase; C, Nessberry, first anaphase. D-J, Nessberry  $\times$  *R. trivialis* F<sub>2</sub>. D, 3n, first anaphase; E, 3n, first metaphase; F, 2n, diakinesis; G, 3n, first metaphase; H, 2n, first anaphase; I, 3n, root tip; J, 3n, first metaphase. K-E, Nessberry  $\times$  Haulsham (*R. idaeus*) F<sub>2</sub>. K, diakinesis; L, second metaphase; M, a, diakinesis; M, b, first metaphase; N, first metaphase; O, first anaphase; P, second metaphase; Q, first metaphase; R, second anaphase. S-T, Nessberry  $\times$  Early Harvest (*R. argutus*) F<sub>2</sub>. 4n, S, first anaphase; T, first metaphase-anaphase.  $\times 2,540$ . Buds and root tips were fixed in Allen's modification of Bouin's solution and paraffin sections made, which were stained in crystal violet. In the buds only pollen mother cells were examined. All drawings were made with the aid of the camera lucida, using a 1.5-mm apochromatic oil-immersion objective and a 12.5  $\times$  compensating ocular.

the seventy-one plants are mentioned in the notes. In four of these the Nessberry parent was considered to be dominant; the fifth had "strong growth, many flowers but infertile, similar in all parts to the F<sub>1</sub>", presumably the intermediate type. The second planting was made October 12, 1927, "Seeds uniformly large." The following February it was noted that the germination was poor, 64 plants being potted. Sixty-eight plants were set in the field during May 1928. Forty-two of these have been available for observation.

The plants may be classified roughly into four groups: (1) A single plant in which the characters of the Nessberry parent are dominant; (2) a variable intermediate group of 10 plants; (3) those almost indistinguishable in appearance of canes from the dewberry parent, 28 plants; and (4) 3 plants similar to those just preceding except that they are more vigorous and show Nessberry characters in the canes. The plants will be discussed in this order.

(1) No. 27 resembles the Nessberry parent very strikingly in type of growth, i. e., the leaflets are large and broad, usually in threes; the canes are strongly branched, with fewer prickles and less pigmentation than the wild dewberry. In addition, the ripe fruit, which is reduced in size because of sterility, crumbles upon picking instead of breaking below the receptacle as in *Rubus trivialis*. Twenty-one chromosomes were observed in root-tip preparations (fig. 2, *I*). There is evidently normal pairing between 14 chromosomes while 7 remain unpaired. This can be seen in the heterotypic anaphase illustrated in figure 2, *D*. Assortment of the unpaired chromosomes is probably at random. Counts of a rather limited number of metaphase plates of the homotypic division indicate that 9 and 12 is the most common distribution, followed by 8 and 13 and then 10 and 11. In figure 2, *L*, the distribution has been 8 to one pole and 13 to the other; one of the chromosomes has split and the halves are about to disjoin.

The 10 plants of the intermediate group (2) differ widely in vigor. Six are highly sterile, the other four having reduced fertility. No. 1, sterile but vigorous, has the tetraploid chromosome number, with complete pairing. Three or four tetrasomes can be observed at diakinesis. Two chromosomes frequently lag at anaphase of the first division. No. 8, sterile and weak, has the diploid chromosome number, which is readily seen at diakinesis (fig. 2, *F*). While the seven pairs seem to behave quite normally, evidence of a loose association between the two components of one of the disomes was noted. No. 12 is fairly fertile; the 14 pairs frequently exhibit a multiple association with from 1 to 6, rarely 7, tetrasomes at diakinesis. The distribution is usually 14 and 14, but 13 and 15 chromosomes are sometimes observed at the homotypic metaphase. Judging from premetaphase and first anaphase stages, plant no. 29 has the tetraploid chromosome number. Chromosomes of certain of the hybrid plants are hard to differentiate clearly, and since no. 29 is one of these, the precise chromosome relationships are hard to determine. There are at least 12 pairs in this case, with a variable amount of multiple association during diakinesis and first metaphase, resulting in 7 or 8 groups of chromosomes in some instances. No. 29 appears to be less fertile than no. 27. The other six plants of this group were not examined cytologically. A summary of chromosome counts for this and other crosses is given in table 1.

An examination has been made of the pollen mother cells from six plants which bear a close resemblance to the dewberry parent (group 3). A count was made of the chromosomes in the root tips of an

additional plant. In each case the diploid number was found. These plants give evidence of their hybrid origin by their vigor, by the immense size of the fruits in many cases, by a peculiar flavor of the fruit of certain of the plants, and in some instances by a reduced fertility. The pairing is complete, although somewhat looser than in *trivialis*. There is a reluctance of one or two chromosome pairs to disjoin promptly (fig. 2, *H*); otherwise the divisions are quite regular. There was a large amount of apparently good pollen—68 and 78 percent for two plants examined in iodine-potassium iodide solution.

TABLE 1.—Summary of chromosome counts obtained

Cross	Group no.	Type	Plants				
			2n	3n	4n	Not examined	Total
			Number	Number	Number	Number	Number
Nessberry × <i>R. rubrisetus</i> (dewberry) F <sub>2</sub>	1	♀ P dominant.....		1			1
	2	Intermediate.....	1		3	6	10
	3	♂ P dominant.....	7			21	28
	4	♂ P predominates, vigorous.....		3			3
		Total.....	8	4	3	27	42
Nessberry × Brighton (raspberry) F <sub>2</sub>	1	Dewberry type.....				2	2
	2	Nessberry type.....			2	4	6
		Total.....			2	6	8
Nessberry × Hailsham (raspberry) F <sub>2</sub>	1	Entirely fertile.....			27	46	73
	2	Fertility reduced.....			28	29	57
	3	Sterile.....			7	16	23
		Total.....			62	91	153
Nessberry × Early Harvest (blackberry) F <sub>4</sub>	1	Entirely fertile.....			18	62	80
	2	Reduced fertility.....			29	38	67
	3	Sterile.....			5	27	32
		Total.....			52	127	179

The last group (4) consists of three plants differing from those immediately preceding by their greater vigor, reduced pigmentation and number of prickles, and increased branching of the canes. All three have the triploid chromosome number. Classification of chromosome groups of an early first metaphase of no. 45b on a basis of size suggests three monosomes, three disomes, and four trisomes (fig. 2, *E*). A spindle view of first anaphase, not illustrated, lends support to this interpretation.

The chromosome number of no. 45c was determined from root-tip material only. Both somatic and meiotic chromosomes of no. 55 were examined. There is a strong tendency for the formation of trisomes in the latter plant, as can be seen during the early first metaphase of figure 2, *G*. There are not usually so many trisomes. In figure 2, *J*, also a first metaphase, there is only one obvious trisome, at the upper right.

#### NESSBERRY × BRIGHTON F<sub>2</sub>

Nessberry × Brighton represents a backcross to a raspberry species, *R. idaeus*, L., similar to the original parent. The first generation was discussed by Ness (9, p. 23). It was noted that—

All of the plants set a profusion of flowers, but not a single flower produced fecund fruit. Each fruit consisted, as in other cases of partial sterility, of only

a few drupelets. By gathering nearly every drupelet, an ample amount of seed for a second generation was secured (9, p. 25).

There were 230 plants, all no doubt with the triploid chromosome number.

Seeds for the second generation were sown in boxes on October 12, 1927; "germination very poor." Eleven plants were potted the following March, and 12 plants were set in the field on May 15, 1928. Eight of these have been available for study. In spite of the use in the breeding work of large numbers of insect-proof cages large enough to cover a plant, it seems highly probable that this family resulted from open pollination, as two of the plants are quite similar to the wild dewberry except in fertility. Three plants of the Nessberry type are almost completely sterile and three plants are quite fertile. Two of the latter have the tetraploid chromosome number. Fourteen pairs can be seen at diakinesis and in both cases there is evidence of a multiple association to give anywhere from one to six loose tetrasomes. Lagging occurs at both heterotypic and homotypic anaphases. One of these had 34 percent apparently good pollen.

#### NESSBERRY $\times$ HAILSHAM $F_3$

Nessberry  $\times$  Hailsham is the same type of cross as the one just discussed, the two first-generation families being very similar. Seed for the second generation was saved from a plant "with perfect, large fruit, foliage differing from rest in texture \* \* \* very similar to Nessberry in all characters." The  $F_2$  consisting of 93 plants, was planted in the field in 1925. Notes were taken the following May on 10 plants. Two were of the Hailsham type, but completely sterile; one was like the wild dewberry, but with "imperfect" fruits; the remainder varied, both in appearance and fertility. On May 16, 1927, an additional plant was described as follows: "Low, compact; leaves, shoots typical of Nessberry; very prolific; flower cluster large; berries large, Nessberry in form and color; drupelets adhering; tendency to break under calyx." While no record is available, this unusual praise, together with the appearance of this third generation, makes it seem quite likely that this plant was its parent. Two hundred and seventy  $F_3$  plants were set in the field on May 14 and 15, 1928. One hundred and fifty-three of these have been available for observation.

With the exception of certain plants of weak growth the family in general favors the Nessberry. All plants have been placed in three groups on a fertility basis, as follows: (1) Entirely fertile, 73 plants; (2) fertility reduced, 57; (3) almost or entirely sterile, 23. The classification is based upon the appearance of the fruit. Buds of 60 plants have been examined. As far as could be determined from pollen mother cell material all plants have the tetraploid chromosome number. The somatic chromosome number of one of these was found to be 28.

While the first group seemed to be completely fertile as judged by achene development, in nine representative plants the percentage of good pollen varied from 45 to 67. In addition, no. 72 had only 16 percent of good pollen, while, in contrast to this, no. 7 had only 3 percent of empty grains, the remainder varying greatly in size, none staining as deeply as pollen from other plants.

Three of the twenty-seven plants examined exhibited no multiple association. Most of the plants had one or two tetrasomes at diakinesis. In four cases as many as seven tetrasomes were sometimes found (fig. 2, *N*), and in one plant six tetrasomes and two disomes. Two of the plants having seven tetrasomes were at first mistaken for diploids (Yarnell (11)), but further examination showed that they as well as the others have the tetraploid number of chromosomes. In spite of an evident reluctance to form a normal plate at first metaphase with consequent lagging of chromosomes during the anaphase following, the usual division is 14 chromosomes to each pole. The great variation in size of pollen grains found in no. 7 is probably due to the loss of lagging chromosomes. The low percentage of good pollen in plant no. 72 was not fully explained by a study of meiosis. There was some evidence of a tendency toward incomplete association at diakinesis but this was rare.

The chromosome behavior of the plants with reduced achene development differs from that of the foregoing group only in degree. The percentage of good pollen from six representative plants was found to vary from 36 to 50, somewhat below that of the previous group. As before, two exceptional plants were found—no. 47 with 85 percent of good pollen and no. 162 with no good pollen.

The diakinesis shown in figure 2, *K*, has 14 pairs of chromosomes. This is from a plant (no. 74) having from none to seven tetrasomes. The figure illustrated in figure 2, *O*, represents a first anaphase of this same plant, and illustrates disjunction without the formation of a metaphase plate. One pair covered by others is drawn outside the cell. The diakinesis of figure 2, *M, a*, has seven tetrasomes, which are shown in figure 2, *M, b*, as they appear at first metaphase. A polar view of the first metaphase of this plant (fig. 2, *Q*) has five tetrasomes and four disomes. Lagging at second anaphase (fig. 2, *R*) occurs in the group.

The last group was originally classified as: (1) Almost sterile, 13 plants; (2) entirely sterile, 3 plants; and (3) weak growth with no flowers, 7 plants. Three plants were found to have the following percentages of good pollen. 3, 21, and 24, respectively. The amount of multiple association varies from none to seven tetrasomes as before. The lack of organization at the normal period for first metaphase is more pronounced, however. This results in nondisjunction more frequently than in the more fertile plants. A 15 and 13 division (fig. 2, *P*) is fairly common.

#### NESSBERRY $\times$ EARLY HARVEST F<sub>1</sub>

Nessberry  $\times$  Early Harvest represents an outcross to a blackberry type. Early Harvest is said to have been propagated from a selected wild plant growing in southern Illinois. It is referred to *Rubus argutus* Link by Card (3) and by Bailey (1). The cross was made in 1919, the small first generation having black fruit rather than the deep red of the Nessberry. In leaves and stem the plants favored the female parent. The flowers were highly sterile. Seeds to produce the second generation were "medium to small, of Early Harvest type." On May 3, 1923, 10 plants were set in the field. On May 19, 1924, there were "seven plants, intermediate except no. 6 and very uniform in characters." One of the intermediate plants was chosen

as parent of the third generation. There was an abundance of both large and small seeds. Sixty-one  $F_3$  plants were set in the field during November 1925. Notes were made on seven of these plants during May 1927. All were fertile and prolific, but they varied somewhat in character of vegetative growth. Seed was saved from two of these for a fourth generation. The seeds were variable in size; germination was good, 336 plants being set in the field in May 1928.

The plants as a whole do not differ greatly in appearance from those of the previous cross, as the Nessberry influence is dominant in both. In each case there is a great deal of variation, particularly in vigor, in size of leaves and flower clusters, and in general habit of growth, including the amount of branching and the degree of procumbency.

A classification of the fertility of the 179 plants available for study was made from the fruit as before. (1) Eighty plants seemed to be entirely fertile; (2) 67 had reduced fertility, and (3) 32 were almost sterile. Pollen was examined from 11 plants of the "fertile" group. They fell into three classes: Six plants with from 70 to 80 percent of good pollen, four plants with from 57 to 59 percent of good pollen, and one plant with 32 percent of good pollen. Ten of thirteen plants examined critically gave evidence of multivalent association, usually having two or three tetrasomes, although two plants had as many as seven tetrasomes part of the time. The other three plants had only disomes at diakinesis and metaphase of the first division. The drawn-out anaphase resulted in an equal distribution of chromosomes in all cases noted. In figure 2, *T*, the chromosomes are separating without the formation of a metaphase plate. There is a single tetrasome at the upper left. This illustrates an extreme degree of irregularity for this group.

Eight of nine plants with "reduced fertility" had from 64 to 82 percent of apparently good pollen. One other plant, no. 23, had only 1.7 percent of good pollen. Buds were fixed from 23 plants of this second group. No. 23 gave evidence of the association of four chromosomes to give two disomes at the metaphase of the second division. From two to four tetrasomes were usually observed during the first division. Anaphase of the first division of this plant was more regular than for most plants of the group (fig. 2, *S*). The chromosomes have been spread in drawing. Considering all 23 plants examined, there were more tetravalent bodies than in the previous group. Only one plant exhibited disomes. The number of tetrasomes commonly found ranged from two to four, with four plants sometimes having as many as six or seven. There was more lagging near the end of the first division in this than in the preceding group. A division of 13 and 15 was observed in six plants; five spores were sometimes formed from one sporocyte.

The six plants classified as "almost sterile" that were examined cytologically differed but little from the foregoing group. The irregularities were of the same type and appeared to be of about the same degree. One plant was found to have 70 percent of apparently good pollen.

#### DISCUSSION

As pointed out by Ness (9) and later by Longley and Darrow (7), the five fertile plants of the second generation of the cross wild dewberry (*Rubus trivialis*)  $\times$  Brilliant red raspberry (*R. strigosus*) behave

as a true species. Since the fertility is due to chromosome doubling in the hybrid  $F_1$ , the Nessberry should be classed as an amphidiploid rather than as a true tetraploid. A second cross made by Ness with the same dewberry by Haymaker, *R. neglectus* Peck (*occidentalis*  $\times$  *strigosus*), appears to have doubled its chromosome complement in a first-generation plant in the same way. Plants of this fertile second generation were fertile with the Nessberry (9).

It is of interest to note that there were only five fertile plants among a second generation otherwise highly sterile. Since the first hybrid generation must have had two genomes of seven chromosomes each it appears that the doubling most likely occurred at a time to produce both egg and pollen grains of an  $F_1$  plant with two genomes—probably in a somatic cell during differentiation of a flower bud, possibly giving a sectorial chimera. If it had happened earlier a larger number of fertile second-generation plants would be expected; if later, sterile  $3n$  plants would result. Some, indeed, may have had the triploid chromosome number. It is possible, of course, that these five plants were produced from the union of nonreduced gametes. This explanation seems to be less likely than the first because seeds from first-generation plants were sown 2 years in succession without the appearance of a single fertile plant.

The four families under consideration represent backcrosses to the two parental species and an outcross to a distinct species, *Rubus argutus* (fig. 1). The first-generation plants of these later crosses would normally be expected to have the triploid chromosome number. This expectation seems to have been realized in each case with the exception of three plants of the backcross to the dewberry, which were fertile and of the maternal type—possibly due to accidental self-pollination.

In two cases plants of a second generation of backcrosses to the two parental species have been available for study. Twenty-eight of the forty-two plants of the cross involving *Rubus rubrisetus* (*R. trivialis*) have a marked resemblance to this species. All of these plants examined are diploid. The large number of plants of this type suggests that they may have originated by the open pollination of  $F_2$  plants with pollen from the wild dewberry, the egg contributing a haploid chromosome set. All exhibit hybrid characteristics and so must contain chromosomes, or at least portions of chromosomes, derived from the original red raspberry parent. In spite of the wide genetic differences between corresponding chromosomes (being derived from different sections of the genus) they are able to pair in these plants and behave otherwise in a fairly normal manner. The raspberry genes are obviously in the minority since the appearance of the plants is in striking contrast to that of the  $F_1$  of the cross *R. trivialis*  $\times$  Brilliant, which also must have been diploid. Ness (9) reports that the raspberry type was very dominant in the  $F_1$ .

The triploids in the backcross under consideration are of two types, one plant being very similar to the Nessberry, and three plants favoring the dewberry but considerably more vigorous. The appearance of these types can be accounted for by supposing that they carry two genomes most or all of whose genes come from the parent they favor. If the latter three plants result from pollen from *Rubus trivialis*, the egg would then have contributed two sets of chromo-



somes, a part of which must have been derived from *R. strigosus*. If association can be taken to represent genetic similarity in this case one would assume that there are two complete genomes and possibly four additional chromosomes that should be referred to *R. trivialis* and three chromosomes deriving largely from *R. strigosus*. In the case of the plant resembling the Nessberry parent one is tempted to assign two genomes to *R. strigosus* and one to *R. trivialis*.

Since the Nessberry regularly has 14 pairs, it is reasonable, considering the wide genetic differences between the parental species, to suppose that pairing is between genomes from the same species, with a resulting lack of opportunity for their modification by crossing over between chromosomes of the two species involved. The first generation of the backcross to the dewberry parent would then have two genomes derived from *Rubus trivialis* and one from *R. strigosus*. Plants with the triploid chromosome number obtained upon selfing a triploid of this constitution would be expected to favor the wild dewberry. The origin of no. 27, favoring the Nessberry is hard to explain. Its appearance, together with the *Drosera* type of pairing observed, strongly suggests that its chromosomal complement does consist of two genomes from *strigosus* and one from *trivialis*.

Plants with the tetraploid chromosome number appear in both second-generation families. This is also probably true in the  $F_2$  of the crosses of Nessberry with the Hailsham raspberry and Early Harvest blackberry, since a portion of plants of these second generations were highly fertile. Selection of plants of the Nessberry type accounts for the appearance, the fertility, and the chromosome number of the later generations studied.

The meiotic chromosome behavior of these later generation 28-chromosome plants differs from that of the Nessberry in two particulars. The association of four chromosomes is characteristic of the heterotypic division and the degree of irregularity is much greater in the former group of plants. The first may result from the accumulation of more than two homologous chromosomes upon the formation of  $4n$  plants from  $3n$  individuals. The second is the natural result of the introduction of chromosomes from a distinct species together with the changed balance just noted.

These conditions account for the variability found in the third and fourth generations under study in regard to general plant vigor and morphological characters. Since these depend upon the particular genetic combination obtaining in each plant and since the chromosomal irregularity is probably dependent upon a more general effect of the chromosome complement, a close correlation between the degree of vigor and chromosome regularity is not to be expected. This seems, in some instances at least, to be true also of fertility and chromosome behavior.

All of the crosses were made by Ness with the object of securing a berry combining the high quality of the red raspberries with the ability of the wild dewberry to withstand conditions of heat and drought, and, of course, the work was done without a knowledge of chromosome behavior in the hybrids. The possibility of chromosome doubling in a hybrid that is highly sterile because of wide genetic differences between the parents is recognized as a means of extending the activities of the plant breeder. The ultimate effect is to complicate the breed-

ing behavior of the material through the use of parents with different chromosome numbers, through the apparent loss of important recessive characters where pairing is only between chromosomes of corresponding genomes in the new amphidiploid, and through the fact that a larger number of chromosomes prevents simple segregation and recombination of characters observed in the original parents. The third and fourth generations of the Nessberry outcrosses discussed above are particularly good examples of the last point. Even in the fourth generation few plants exhibit a really stable chromosomal balance. Practically all characters show some influence of both parents. Where such chromosome doubling has occurred, the breeder may expect a high degree of variability both as to degree of fertility and general somatic characters in using the material in outcrosses to secure more desirable combinations.

#### SUMMARY

A cytological study has been made of crosses involving the amphidiploid hybrid of a dewberry  $\times$  red raspberry cross (the Nessberry) with the diploid dewberry parent and with two diploid raspberries and a blackberry.

Plants with the diploid, triploid, and tetraploid chromosome number were found in the second generation of the dewberry backcross. A single diploid was of intermediate type; 28 diploids strongly favored the dewberry type and may represent outcrosses of the  $F_1$  to the wild species. The triploids were of two types, one plant favoring the Nessberry and three plants nearer to the wild dewberry but more vigorous. Seven disomes plus seven single chromosomes were found during the first division of the first triploid; from three to four trisomes (occasionally more) can be distinguished in the second type.

Plants of the third generation of the Nessberry  $\times$  Hailsham red raspberry cross and plants of the  $F_4$  of the Nessberry  $\times$  Early Harvest blackberry cross all had the tetraploid chromosome number. This is probably due largely to selection of plants favoring the Nessberry in the second and third generations. Meiosis of these plants is distinguished from that of the Nessberry by the frequent occurrence of multivalent association to give from one to as many as seven tetrasomes, and by considerably greater chromosomal irregularity during the reduction division.

It seems likely that such association reflects genetic similarity among the associating chromosomes, since there has been an opportunity for more than two corresponding chromosomes of a genom to become segregated in a single plant.

A wide variation in vigor observed among the plants of these later generations is probably due to genetic differences possible in the recombination of chromosomes from the three species involved.

There was some correlation between chromosome regularity at meiosis and degree of fertility, but the most sterile plants did not exhibit a greater degree of chromosome irregularity than many less sterile.

It is pointed out that a doubling of the chromosomal complement in a sterile hybrid greatly complicates its breeding behavior in subsequent outcrosses.

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## BLACK CHAFF DISEASE OF WHEAT<sup>1</sup>

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### INTRODUCTION

Black chaff of wheat (*Triticum aestivum* L., *T. vulgare* Vill.), caused by *Bacterium translucens* var. *undulosum* Smith, Jones, and Reddy, has been present in the wheat-growing region of the Mississippi Valley for a number of years. Although it has become distributed generally throughout the central and upper Mississippi Valley, it usually does not become epidemic over large areas on the varieties commonly grown. This sporadic occurrence of severe infection may be due to variations in environment. No extensive investigations have been made to determine what environmental conditions favor the development of epidemics of the disease, although the heaviest infections have occurred in wet seasons. In the hard red spring wheat area Hope and H-44, two varieties that have proved very resistant to stem rust, are being used extensively as parent stock in the breeding of new disease-resistant varieties. Since these two varieties are among the most susceptible to black chaff, there is danger of the disease becoming epidemic under favorable environmental conditions should selections obtained from crosses with these varieties, or with other susceptible ones, become widely grown. Therefore, it is evidently desirable to know the susceptibility or resistance of the new varieties and hybrid selections grown. However, in order to test most effectively varieties and hybrid selections for their reaction to black chaff by means of artificial epidemics, it is necessary to know the environmental conditions most favorable for the production of an epidemic and the life history and physiologic specialization of the pathogen.

### GEOGRAPHIC DISTRIBUTION AND ECONOMIC IMPORTANCE

The black chaff disease of wheat is widely distributed, occurring rather generally in many of the principal wheat-producing countries of the world. In North America it is known to occur in the United States, Canada, and Mexico. The first record of its presence in the United States was probably made by Heald (10)<sup>3</sup> in 1906, when he

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<sup>2</sup> The writer gratefully acknowledges his indebtedness to the following members of the staff of the Minnesota Agricultural Experiment Station: Dr. E. C. Stakman, under whose direction these investigations were made, Dr. J. J. Christensen for suggestions and criticism during the progress of the work, and Dr. J. G. Leach for help in the preparation of the manuscript.

<sup>3</sup> Reference is made by number (italic) to Literature Cited, p. 416.

briefly called attention to a bacterial blight of the leaves of wheat in the breeding plot at the Nebraska Agricultural Experiment Station that year. Since that time it has been found throughout the Mississippi Valley and in California (19).<sup>4,5</sup> Bisby and Buller (2) first reported the presence of black chaff in Canada in 1922. Drayton (5) stated that it occurred there in 1923 and 1924, and Greaney (8) found it in Manitoba every year from 1926 to 1929. During the summer of 1931 the writer received from Mexico specimens of wheat affected by a disease which he identified as black chaff. The specimens were collected by H. G. Ukkelberg in the State of Coahuila. Other specimens were received in April 1933 from Wallace Butler from the State of Nuevo León.

Jaczewski (12) first reported black chaff in Europe in 1925. He found it on wheat samples collected as early as 1910 and 1911 from the Provinces of Mohilev in Belorossia and Elisavetopol in Crimea. He also found it on samples from the Province of Poltava in Ukraine collected in 1916, from Crimea in 1922, and from Kharkov and Kiev in 1924. It was also found in the Provinces of Voronezh and Don, and the district of Kuban in Caucasia. Millasseau (16) first noted the disease in France in 1927. He found it rather generally distributed in the area around Paris. The next year (1928) Hocquette (11) found it at Cappelle, in the Department of Nord, which is adjacent to Belgium, and Marchal (15) found it in Belgium in 1929.

Jaczewski (12) gave evidence of the occurrence of the disease in Asia as early as 1893. He found black chaff on specimens of wheat collected in Sungpan, China, in that year. No other reports of the disease in Asia have been found.

The first report of the occurrence of black chaff in Africa appeared in 1930. Verwoerd (22) found a bacterial disease on wheat in the plots at the College Cereal Experiment Station, Malmesbury, South Africa, in 1929, which he later identified as black chaff. Burton (4, *Rept. 1930*) noted the occurrence of the disease in Kenya, British East Africa, in 1930.

Published data regarding the losses in yield and quality of grain due to black chaff are limited. The reports that have appeared have mostly been estimates of losses over large areas without experimental data giving comparative yields of infected and uninfected plants. Burton (4, *Rept. 1931*) reported that a number of varieties in yield trials in 1931 were completely destroyed by the disease. Waldron (23) made a comparison between the weight of grain from infected and noninfected plants in a number of  $F_3$  families from crosses of Hope with other varieties. He found that there was a decrease in the weight of the grain from the infected plants and that as an average of several families the decrease was 10.5 percent. Evidently, the amount of loss that would be caused by the disease depends upon the time and severity of infection. The writer has found that where there is severe infection part of the spikelets are often barren and the more severely infected culms may be entirely barren.

#### HISTORICAL REVIEW

As early as 1906, Heald (10) recorded the occurrence of a bacterial blight of wheat in the breeding plot at the Nebraska Agricultural

<sup>4</sup> SMITH, E. F. BLACK CHAFF OF WHEAT. U. S. Dept. Agr., Bur. Plant Indus. Plant Disease Survey Bull. 2: 98-99. 1918. [Mimeographed.]

<sup>5</sup> HASKELL, R. J., and WOOD, J. I. DISEASES OF CEREAL AND FORAGE CROPS IN THE UNITED STATES IN 1928. U. S. Dept. Agr., Bur. Plant Indus. Plant Disease Survey Bull. 71: 275-278. 1929. [Mimeographed.]

Experiment Station. Although he gave no description of the diseased except to say that it was a bacterial blight of the leaves, it probably was black chaff. Jones, Johnson, and Reddy in 1916 (13) called attention to a bacterial blight of wheat, which they found to be similar to or identical with a disease of barley that they called bacterial blight. They stated that the symptoms of the two diseases and the cultural characters of the causal organisms appeared to be identical. Although their description of the symptoms of barley blight was brief, it was accurate enough to leave no doubt that the disease which they found on wheat was the same as that later described by Smith (19) as black chaff.

Smith (19), in 1917, described the disease very clearly as it appears on the glumes of wheat, especially on ripening grain, but he did not point out definitely the symptoms on seedlings or on the parts of older plants other than the glumes. In a note written later in the same year he reported that he had been able to obtain a high percentage of seedling infection with artificial inoculations and that he had isolated the pathogen from diseased leaves, stems, glumes, and kernels.<sup>6</sup> He found bacteria in great numbers in corroded cavities in the interior of shriveled grain and he believed that the general appearance of the pathogen in and on the wheat grains suggested that the organism might live over from one year to the next on infected seed. The following year (1918) he obtained what he considered evidence substantiating this belief and suggested the following methods of ridding the seed of this hold-over inoculum:<sup>7</sup> (1) Screening to remove the shriveled grain containing the bacterial cavities and (2) treating the seed grain with 1:1,000 copper sulphate solution to destroy the surface bacteria.

Braun (3) presented very strong evidence that seedling infection occurred in Kansas in 1919 in fields planted to seed from infected fields, and that the seedling infection there was greatly reduced or entirely eliminated by treating the infected seed with formalin or copper sulphate.

Although the bacterial nature of the black chaff pathogen had been known for several years, no description of the organism or its host range was available before that given by Smith, Jones, and Reddy (20) in 1919. In morphology, physiology, and cultural characteristics they found it to be almost identical with *Bacterium translucens*, the pathogen of bacterial blight of barley, as described by Jones, Johnson, and Reddy (14). The principal difference between the wheat and barley organisms was in pathogenicity. In cross inoculations on the leaves of seedlings the barley pathogen was either noninfectious on wheat or produced small nontypical lesions, while the wheat pathogen was almost as pathogenic on barley as on wheat and the lesions produced on barley by the two organisms were indistinguishable.

#### OBJECTS OF THE INVESTIGATIONS

The specific objects of these investigations were to study: (1) The factors which contribute to the production of an epidemic, (2) the life history of the causal organism, (3) the method of overwintering

<sup>6</sup> SMITH, E. F. BLACK CHAFF OF WHEAT. U. S. Dept. Agr., Bur. Plant Indus. Plant Disease Survey Bull. 1: 40, 1917. [Mimeographed.]

<sup>7</sup> SMITH, E. F. See footnote 4.



of the organism, (4) varietal susceptibility, and (5) the specificity of pathogenicity of the causal organism.

#### MATERIAL AND METHODS

In the fall of 1929, a culture of the pathogen of black chaff of wheat was obtained from Dr. Delia Johnson, who had made the isolation from diseased wheat grown at University Farm, St. Paul, Minn., in 1929. For convenience this isolate has been called culture M. Another culture was received from A. G. Johnson, of the Division of Cereal Crops and Diseases, Bureau of Plant Industry, United States Department of Agriculture, Washington, D. C. This was designated as culture W.

During the early summer of 1931 sectors appeared in old colonies of cultures M and W growing on potato-dextrose agar in Erlenmeyer flasks. These sectors differed from the original colonies in color, viscosity, and rate of growth. Transfers were made from these sectors by removing a small amount of bacterial growth from the margin to fresh agar slants with the end of a flamed needle. With this procedure three new cultures of the organism were obtained, one as a sector from culture M and two as sectors from culture W. These were designated as M-1, W-1, and W-2, respectively.

The culture medium used for stock cultures and all experimental work, unless otherwise stated, was potato-dextrose agar (1 percent of dextrose and 1.5 percent of Bacto-agar).

Stock cultures of the black chaff organism were kept on agar slants, either at room temperature in the laboratory or in an ice box. Transfers were usually made at 2- to 4-week intervals to keep a supply of vigorously growing cultures on hand. After being kept in the laboratory for approximately 3 weeks during parts of July and August 1931, cultures M and W failed to grow when transferred to fresh agar slants, so these were no longer available for experimental work. All efforts to revive them with various culture media failed. However, cultures M-1, W-1, and W-2 did resume growth when transferred to fresh agar slants and these were used as inoculum for later pathogenicity and cultural studies.

Water suspensions of the bacteria were used as inoculum. These suspensions were made by scraping into tap water the bacterial growth from fresh colonies growing on agar slants. Usually cultures 72 to 96 hours old were used, and enough of the bacterial growth was put into the water to make a heavily clouded suspension.

When the writer began work on black chaff in the fall of 1929 no method of inoculating the plants with the pathogen had been described which was suitable for greenhouse experiments. Therefore, one of the first problems was to develop a method of inoculation for such work. Three different methods of inoculation were used: (1) Spraying the plants thoroughly with the bacterial suspension by means of an atomizer; (2) rubbing the suspension on the leaves with the fingers; (3) forcing the inoculum into the leaf roll of young plants or into the boot of older plants with a hypodermic syringe.

Spraying plants with suspensions of the bacteria in water or nutrient broth usually gave poor results, even when the plants were kept in moist chambers for 48 hours after inoculation. This was probably due to the fact that the inoculum which remained on the surface of

the plants and even the bacteria which had entered the substomatal cavities were quickly dried out and their further advance into the host checked by the low humidity existing in the greenhouse in cold weather. On the other hand, it was sometimes desirable to place these plants immediately after inoculation, without incubation in moist chambers, under the conditions in which they were to be grown. In such cases no infection resulted from the spraying method of inoculation. Likewise, when the inoculum was rubbed on the leaves with the fingers the results were either negative or not consistent enough to be reliable.

The most successful method found was that of introducing the inoculum into the leaf roll by means of a very small needle on a hypodermic syringe. The injections were made by sticking the point of the needle into, but only partially through, the leaf roll at an angle, the beveled side of the point being held toward the plant. In this way one or more injections could be made with little injury to the plant. At each injection several punctures were usually made through one or more of the rolled leaves, thus injuring several surfaces for the bacteria to attack. Whether seedlings or older plants were used, a quantity of inoculum could be forced between the rolled leaves and even out of the top of the spiral. Thus the leaf roll served as a moist chamber, protecting the inoculum from desiccation. Hereafter this method will be referred to as "hypodermic inoculation." It was the method used for all experimental inoculations unless otherwise stated.

## ETIOLOGY

### DESCRIPTION OF CAUSAL ORGANISM

Black chaff of wheat is caused by *Bacterium translucens* var. *undulosum* Smith, Jones, and Reddy. This organism is a monotrichous rod; hence the genus corresponds to Migula's *Pseudomonas* and the Society of American Bacteriologists' *Phytomonas*. Smith, Jones, and Reddy (20) did not give in detail the physiologic and cultural characteristics for this new variety but stated that in general they were the same as those described for *Bact. translucens*.<sup>5</sup> The only exception noted was that on whey agar the slime produced by the wheat pathogen was a deeper yellow and less fluid than that produced by the barley pathogen. This difference might not have held if they had made comparative studies with a number of isolations of the two organisms. The writer found wide variations in the color and viscosity of the bacterial growth among the five cultures (M, W, M-1, W-1, and W-2) within the variety *Bact. translucens undulosum*. On agar slants culture M was slightly deeper yellow and produced less fluid slime than culture W. However, M-1 was very light yellow and produced little slime. Cultures W-1 and W-2 were very similar to their parent culture, W, except for a slightly deeper yellow color. Since the writer found that color of colonies and fluidity of slime are not constant characters with *Bact. translucens* var. *undulosum*, he does not consider these characters a good basis for differentiating between *Bact. translucens* and *Bact. translucens* var. *undulosum*.

Two other differences between the two organisms were described by Godkin (6). He found that within the colonies of both bacteria

<sup>5</sup>JOHNSTON, C. O. DISEASES OF WHEAT IN PENNSYLVANIA AND KANSAS. U. S. Dept. Agr., Bur. Plant Indus. Plant Disease Repr. 13: 86. 1920. [Mimeographed.]

there were minute undulations or striations, as had been previously described for *Bacterium translucens* var. *undulosum* (20), but that they were more pronounced and appeared sooner in colonies of *Bact. translucens* var. *undulosum* than in colonies of *Bact. translucens*. The writer was unable to get authentic cultures of *Bact. translucens* to make comparisons with *Bact. translucens* var. *undulosum* in this respect.

The other difference which Godkin found between the two organisms was in their action on lactose. He stated that, in beef bouillon plus lactose, *Bacterium translucens* var. *undulosum* produced an acid reaction while *Bact. translucens* produced an alkaline reaction. These results are only in partial agreement with the results obtained by the writer. When grown on beef bouillon plus lactose, culture W and cultures W-1 and W-2, which arose as sectors from culture W, either produced an acid reaction or caused no change in the reaction of the medium. In two out of three trials culture W caused no change of reaction in the medium, while in the third trial it produced an acid reaction. No change of reaction was evident in the single trial with W-1 and W-2. In all the trials with sugars, from 6 to 10 tubes of the medium were inoculated in each case, and 4 tubes were left sterile as checks. On beef bouillon plus lactose, cultures M and M-1 produced an alkaline reaction or no change. In two out of three cases there was no change in the reaction caused by M, but in one trial with M and in the single trial made with M-1 there was a definite change to alkalinity. To the writer, these conflicting results indicate that *Bact. translucens* var. *undulosum* does not cause a specific reaction when grown on beef bouillon plus lactose, but rather that certain isolations of the organism may produce acid while others produce alkali. It is not strange that isolations of this organism should differ in this characteristic, because, as will be shown later, they differ in other respects. Since neither Jones, Johnson, and Reddy (14) nor Smith, Jones, and Reddy (20) recorded a difference in action on lactose between the two organisms, it seems likely that in this respect the cultures with which they worked were very similar to each other but different from the cultures used by Godkin.

#### RELATION OF ORGANISM TO HOST TISSUE

The writer was unable to find any report of work having been done to determine how the pathogen enters the host or what part of the tissues it attacks. Millasseau (16) made the statement that the bacteria are within the tissues of the host in older lesions, but he did not say whether they were intercellular or intracellular. Jones, Johnson, and Reddy (14) found that infection by *Bacterium translucens* was stomatal and that the bacteria invaded the intercellular spaces of the parenchyma in the early development of lesions. Instances of advanced invasion were found where the cell walls had partially disappeared. Their results are very similar to those obtained by the writer with *Bact. translucens* var. *undulosum*.

*Bacterium translucens* var. *undulosum* enters the host plant by way of the stomata and through wounds. Fresh leaves from inoculated wheat seedlings were dipped for a few minutes in a staining solution made up of 0.1 percent of thionine in 50-percent alcohol. The seedlings had been inoculated by spraying them with a water suspension of the bacteria and had been kept in moist chambers either 24 or 48

hours before being used. Examination of these leaves with the low power of a microscope showed masses of deeply stained material just beneath many of the stomata. When razor sections were cut from the infected areas and examinations made with higher magnification, these masses were found to be bacteria. Some of the bacteria had evidently got through the stomata and multiplied in the substomatal cavities. Many bacteria were found on the surface of the epidermis of the sprayed leaves, but there was no sign of entrance and multiplication except beneath the stomata. However, where punctures had been made masses of bacteria were found around the margin of the punctures, showing that they may also attack the host tissues in places where the epidermis is broken.

In order to find out what part of the tissues was attacked older lesions were embedded in paraffin and sectioned. The sections were stained with thionine and orange G. In these lesions the bacteria were both intercellular and intracellular. There were masses of bacteria in many of the parenchyma cells, but the vessels appeared to be free from invasion. In the infected areas the intercellular spaces were filled with bacteria and part of the cells were crowded out of shape. Some of the cell walls were completely broken down and the cell contents replaced with masses of bacteria, but bacteria were observed also in certain other cells the walls of which apparently were not greatly injured.

#### SYMPTOMS OF THE DISEASE

The most conspicuous signs of the disease appear on the glumes and awns, but Smith (19) has already given a good description of this phase of the disease. However, his description of the lesions on the leaves, culms, and rachises was too brief and indefinite to be of much use in recognizing the disease. Therefore, the symptoms on these parts are given.

#### LEAVES

The first evidence of the disease on the leaves is very small, water-soaked, dark-green spots which may be on the midrib or on any part of the leaf blade. On plants that have been artificially inoculated by forcing the inoculum into the leaf roll with a hypodermic needle, the infection usually appears first at the margins of the punctures made by the needle. On plants that have been inoculated by spraying inoculum on the leaves or by rubbing it on with the fingers and keeping the plants in moist chambers for 24 hours, the infection spots result from stomatal infections. These infection spots enlarge with the progress of the pathogen through the host tissues, mostly longitudinally, thus giving the older lesions an irregularly streaked appearance. The leaf veins usually check partially or entirely the lateral advance of the pathogen. The lesions may be limited by two adjacent leaf veins or they may involve the entire width of the leaf, in which cases those parts of the leaf beyond the infected areas soon wilt and die. The infection may progress only a millimeter or two lengthwise of the leaf, or it may extend out to the tip of the leaf or down to the ligule and even down the leaf sheath. The infected tissues gradually lose their water-soaked dark-green color and become more transparent and chlorotic. Older lesions often become dark yellow or brown and may be streaked with black. Entire lesions

sometimes become black. Under moist conditions exudate appears on the fresh lesions and may dry down into a thin scale or into small hard yellowish beads.

#### CULMS AND RACHISES

Light brown to black sunken streaks appear on the rachises and on the necks of the culms for some distance below the spikes. These may be narrow and scattered around the culm or they may coalesce and darken the whole circumference of the stem. Similar lesions may appear just below the nodes, especially the lower nodes, of the culms. These infected areas near the nodes appear as discolored bands and may be either entire or made up of many narrow streak lesions.

Apparently the appearance of the disease may vary on different varieties of wheat. Some varieties, especially Hope and H-44, are frequently attacked at or near the lower nodes of the culms without showing the disease on the heads and necks. In other cases, infection on these varieties may be general throughout the length of the culm. Other varieties are infected much more commonly on the necks and floral parts without any nodal infection.

### GREENHOUSE AND LABORATORY EXPERIMENTS

#### INFLUENCE OF ENVIRONMENT ON DEVELOPMENT OF THE ORGANISM IN CULTURE

##### TEMPERATURE

As a preliminary step to the determination of the temperature at which the black chaff organism was most virulent, it was thought desirable to find the cardinal temperatures for its growth on culture media. Petri dishes 100 mm in diameter, containing 20 cc of agar, were inoculated in the center from a fresh transfer of culture M. Care was taken to get as nearly as possible the same amount of inoculum on each agar plate. Ten plates were then put at each of several temperatures from 0° to 40° C. The relative growths at the different temperatures were measured by taking the mean diameters of the colonies on three different dates. A few contaminated plates were discarded, but in no case were there fewer than eight colonies for measurement at any temperature. The experiment was repeated within a few days, and the average diameters of the colonies grown at the different temperatures are given in table 1.

TABLE 1.—Effect of temperature on growth of *Bacterium translucens* var. *undulosum* on potato-dextrose agar

Temperature (° C.)	Col- onies	Average diameter of colonies in—			Temperature (° C.)	Col- onies	Average diameter of colonies in—		
		2 days	4 days	7 days			2 days	4 days	7 days
0.....	Number	Mm	Mm	Mm	25.....	Number	Mm	Mm	Mm
10.....	19	0.00	0.00	0.00	30.....	17	1.80	4.74	7.65
15.....	18	.00	.10	.44	35.....	19	2.19	5.65	8.74
20.....	17	.00	.63	1.62	40.....	18	.48	1.02	.98
	18	.67	3.39	6.03		20	(1)	(1)	-----

<sup>1</sup> Very slight growth on only 3 plates.

From the results given in table 1 it is evident that *Bacterium translucens* var. *undulosum* grows best in culture at a high temper-

ature, probably slightly below 30° C., and also that it grows rapidly at temperatures ranging from 20° to 30°. The minimum temperature at which it will grow is somewhat less than 10° and the maximum approximately 40°.

Very small colonies appeared on some of the plates kept at 40° C. and remained for a few days, but by the seventh day the agar in the dishes had dried out so much at this high temperature that the colonies also had almost entirely dried up.

#### HUMIDITY

The fact that black chaff is more prevalent and that the pathogen progresses through the tissues of the host more rapidly in wet weather than in dry suggested the possibility that humidity might be one of the controlling factors in the development of the disease. Experiments were made to determine the effect of different degrees of relative humidity of the surrounding atmosphere on the growth of the organism on culture media. Fresh agar slants were inoculated by touching the end of a flamed needle to a 48-hour-old colony of W-2 and then touching the adhering bacteria to the agar. Five tubes were kept at each of several constant relative humidities between 10 and 100 percent. The constant humidities were maintained by the use of aqueous solutions of sulphuric acid of known specific gravity. The different concentrations of acid for the maintenance of relative humidity of different percentages were made up according to the table given by Stevens (21). Seventy-five cubic centimeters of each concentration of acid were put in the bottom of rectangular glass dishes, approximately 9 by 19 cm and 6 cm deep. The inoculated test tubes were then put in the dishes above the acid; glass slides on Syracuse dishes were used to keep the tubes out of the acid. The loose cotton plugs were removed from three of the five tubes before the covers were sealed on the dishes with vaseline. The cultures were grown at laboratory temperature (approximately 21° to 25° C.). The writer realizes that the bacteria themselves were not exposed to the relative humidity maintained in the surrounding atmosphere and that the effects on growth may have been a function of the rate of drying. Since there was no noticeable difference in rate of growth between the colonies in the tubes with and without the loose cotton plugs, no differentiation is made in table 2.

TABLE 2.—Effect of humidity on the growth of *Bacterium translucens* var. *undulosum* in culture

Relative humidity (percent)	Relative growth <sup>1</sup> at—			Relative humidity (percent)	Relative growth <sup>1</sup> at—			Relative humidity (percent)	Relative growth <sup>1</sup> at—		
	Laboratory temperature <sup>2</sup>	22° C.	27° C.		Laboratory temperature <sup>2</sup>	22° C.	27° C.		Laboratory temperature <sup>2</sup>	22° C.	27° C.
100.....	5	-----	-----	60.....	5	4	5	30.....	2	0	0
90.....	5	-----	-----	50.....	4	3	4	20.....	-----	0	0
80.....	5	-----	-----	40.....	-----	2	4	10.....	1	0	0
70.....	5	-----	-----								

<sup>1</sup> The numbers 0 to 5 designate the relative amounts of bacterial growth at the different percentages of relative humidity.

<sup>2</sup> Approximately 21° to 25° C.

Since there were no differences in rate of growth on the medium at relative humidities of 60 percent or above and no cultures were held at the humidities of 40 and 20 percent, the experiment was repeated with humidities of 60, 50, 40, 30, 20, and 10 percent and constant temperatures of 22° and 27° C. Eight cultures were used in each dish in these trials, and the cotton plugs were removed from all after they had been placed in the dishes. Table 2 shows the relative amounts of growth on the agar slants at these temperatures and relative humidity percentages after 10 days.

It is evident from these results that a high relative humidity of the air surrounding the colonies is conducive to the most rapid growth of the black chaff organism on culture media. It is also evident that very low relative humidities may greatly retard or prohibit the development of colonies.

#### INFLUENCE OF TEMPERATURE ON INCUBATION PERIOD

The time required for the appearance of the first signs of black chaff after inoculation varied with the conditions under which the plants were kept. The usual incubation period for the disease on wheat seedlings was from 5 to 8 days when the temperature of the greenhouse was normal (about 18° to 22° C.) and the plants were kept in moist chambers for 48 hours after inoculation, but under optimum conditions for the development of the bacteria the incubation period was shortened. On at least two different occasions, when the temperature of the greenhouse was much higher during the day, well-defined lesions were present when the plants were removed from the moist chambers 48 hours after inoculation. When the plants were kept in a controlled-temperature room at 10°, the earliest signs of infection appeared 8 days after inoculation, and the average incubation period was from 15 to 20 days. At a controlled temperature of 20°, the earliest signs of infection appeared in 2 days after inoculation, and the average incubation period was from 5 to 7 days.

#### INFLUENCE OF ENVIRONMENT ON ADVANCE OF THE PATHOGEN AND ON SYMPTOMS

The length of the period of incubation is not the only phase in the development of the disease which is affected by environment. The rate of advance of the pathogen through the host tissues, the total amount of the host tissue finally affected, and the appearance of the older lesions are also affected by the conditions under which the plants are grown.

#### TEMPERATURE

A series of experiments were made in which the plants were placed at different temperatures and different light intensities immediately after inoculation. Hope and Kanred plants were used in the experiments, but, since there were no appreciable differences between these varieties in percentage of plants infected or in size and appearance of the lesions, the varieties are discussed as one. The conditions under which the plants were tested after the inoculation were as follows: (1) One series of the plants was kept in a controlled-temperature room at 10° C. and lighted by a single 1,000-watt lamp placed 6 to 8 feet away from the plants; (2) another series was kept at a controlled temperature of 20° and lighted by a single 1,000-watt

lamp placed 8 to 10 feet from the plants; (3) the remainder of the plants, immediately after inoculation, were moved to a greenhouse bench where they received all the available sunlight and where the temperature varied from approximately 20° to 30°. The amount of infection obtained in the several trials under the three sets of conditions is summarized in table 3.

TABLE 3—*Effect of temperature and light during incubation period on infection of wheat by Bacterium translucens var. undulosum*

Environmental conditions			Plants inoculated	Plants infected			Length of lesions on seedling plants		Notes on adult plants
Place	Temperature	Lighting					Average	Maximum	
	° C		Number	Number	Percent		Mm	Mm	
Control chambers.	10	1,000-watt lamp at distance of 6 to 8 ft.	235	176	74.8		1-2	4-5	Lesions showed no black striping and no darkening. One plant showed slight pigmentation of lesions on 2 or 3 glumes after 30 days.
	20	1,000-watt lamp at distance of 8 to 10 ft.	214	187	87.3		10-15	20-30	
Greenhouse....	20-30	All available sunlight	324	264	81.4		-----	(?)	Lesions showed typical dark-brown or black striping within 14 to 21 days.

<sup>1</sup> Long water-soaked infected areas in 30 days.

<sup>2</sup> Some lesions extended entire length of leaves.

From the results obtained from these experiments the writer concluded the temperature at which the plants are grown is not one of the most important factors determining the percentage of plants that become infected. It can be readily seen from table 3 that there are no striking differences in the relative number of plants infected at 10° or 20° C. or at higher temperatures in the greenhouse. However, the size of the lesions or the amount of host tissue attacked by the pathogen within a given time was influenced by temperature. At 10° the infected areas on seedling plants were very small even after 30 days; the average lesions were only 1 to 2 mm long, and the longest only 4 to 5 mm. At 20°, on plants of the same age, there were long water-soaked infected areas in 30 days, the lesions averaging 10 to 15 mm in length, with some as long as 20 to 30 mm. In the greenhouse some of the lesions on the plants extended the entire length of the leaves.

#### LIGHT

Light intensity plays a role in determining the appearance of the older lesions on diseased plants. Lesions on adult plants kept in the controlled-temperature chambers at 10° and 20° C. under artificial illumination did not show the black striping typical of the lesions on plants approaching maturity in the field. One plant out of more than 100 that had been kept in the 20° room for 30 days showed a slight pigmentation of the lesions on two or three glumes. At 10° there was no darkening of the lesions on any of the plants. Almost all the



lesions on adult plants kept on the greenhouse bench after inoculation showed the typical dark-brown or black striping within 14 to 21 days.

#### VIABILITY OF THE BACTERIA UNDER ADVERSE CONDITIONS

Although Smith and his associates (20) suggested that one source of inoculum for spring infection might be bacteria found in the refuse and stubble left on infected fields, they presented no evidence that the bacteria could withstand the adverse conditions to which they would be subjected under natural conditions out of doors during the winter and early spring before host plants were available for attack. In order to find out whether the black chaff organism could survive the adversities of winter, the writer made some experiments during the winter and spring of 1932-33.

Forty-eight-hour-old colonies of culture W-2 on agar slants were put outside on the ground in an exposed place on December 15. Transfers to fresh agar slants were made from these colonies on December 30. In every case vigorous growth, which appeared to be the same as the original culture, appeared on the fresh agar slants. Later pathogenicity tests proved that the new growth was *Bacterium translucens* var. *undulosum*. During the time the cultures were exposed the temperature ranged from  $-27.8^{\circ}$  to  $11^{\circ}$  C., showing that freezing temperatures do not quickly kill the bacteria, at least when on artificial media. Other cultures on agar were put under similar conditions December 15, 1932, and transfers were made at intervals up to April 18, 1933. Transfers made on that date, 124 days after exposure, grew vigorously.

In order to find out whether the bacteria could withstand adverse conditions approximating those to which they are subjected in wheat fields during the winter, the following experiment was made. Bacterial growth from 48-hour-old cultures of W-2 was transferred to a number of small Erlenmeyer flasks half filled with steamed soil, the steaming having been done in the autoclave at 15 pounds pressure for 1 hour after the soil had been put into the flasks. Some of the flasks contained dry soil and some wet. The soil inoculations were made by placing one loop of the bacterial growth into each flask with a flamed needle and shaking the flask until the bacteria were thoroughly mixed through the soil. Some of these flasks were put outside and exposed to cold and changing temperatures on December 15, and some were kept in the laboratory. At irregular intervals, beginning 2 days after inoculation, one flask each of the wet and of the dry soil was brought into the laboratory and transfers were made from them to agar slants. Transfers were also made from the flasks kept in the laboratory, by removing with a flamed needle one or two loops of the soil from each flask to the agar. Table 4 gives the results of the transfers after different lengths of exposure and shows definitely that *Bacterium translucens* var. *undulosum* may remain viable through a long period of continued exposure to low temperatures and to alternate freezing and thawing, conditions so detrimental to many micro-organisms. During the 124 days the cultures were left outside, the temperature ranged from  $-33.3^{\circ}$  to  $20^{\circ}$  C.

Other soil cultures of the organism were put in a controlled-temperature room at  $-12^{\circ}$  C. on January 31, 1933, and transfers were made from these at intervals until April 18. Vigorous growth was

obtained from these transfers on every date they were made. During the same period other soil cultures were kept in the laboratory, where the relative humidity was very low. Since the flasks were covered only with cotton plugs, the soil became very dry. However, bacteria remained viable in the dry soil throughout the 77-day period.

TABLE 4.—*Viability of Bacterium translucens* var. *undulosum* in soil cultures after exposure to normal temperatures<sup>1</sup> out of doors between Dec. 15, 1932, and Apr. 18, 1933, at St. Paul, Minn., as determined by transfers to agar slants<sup>2</sup>

Date of transfer to agar slant	Days exposed	Condition of soil		Date of transfer to agar slant	Days exposed	Condition of soil	
		Moist	Dry			Moist	Dry
1932				1933			
Dec 17.....	2	+	+	Jan. 6.....	22	+	+
Dec 20.....	5	+	+	Feb. 6.....	53	+	+
Dec 30.....	15	—	—	Mar 21.....	96	+	+
				Apr. 18.....	124	+	+

<sup>1</sup> Ranging from  $-33^{\circ}$  to  $20^{\circ}$  C.

<sup>2</sup> Plus and minus signs denote whether or not transfers from soil cultures to agar slants grew.

These results indicate that *Bacterium translucens* var. *undulosum* is able to withstand adverse environmental conditions for considerable periods of time.

## FIELD OBSERVATIONS AND EXPERIMENTS

### OCCURRENCE OF THE DISEASE IN RELATION TO AGE OF HOST

Black chaff of wheat does not ordinarily appear in Minnesota until the plants are headed out. The progress of the disease was carefully observed in experimental plots at University Farm during 1930, 1931, and 1932. In an extensive field trial at Abilene, Kans., in 1919 Braun (3) observed as high as 10.5 percent seedling infection in a field of Kanred wheat that had been sown with seed from a field heavily infected with black chaff the year before. The writer has been unable to find any evidence of infection on wheat plants before they had reached the booting stage, except on plants that had been inoculated experimentally. Seed saved from plants heavily infected with black chaff in 1930 and 1931, was sown the following years (1931 and 1932) in plots parallel with plots sown to seed from uninfected plants. Thousands of plants were examined, but no seedling infection was found in these plots either in 1931 or in 1932. Later in the season, after the plants had headed, some black chaff developed, but there was no noticeable difference in the amount of infection in the plots from infected and noninfected seed. During the winters of 1931-32 and 1932-33 seed saved from infected plants and seed inoculated with cultures of the black chaff organism were planted in pots in the greenhouse. These pots were kept in moist chambers until the plants were 3 or 4 inches high, but no signs of black chaff infection appeared.

### ENVIRONMENTAL INFLUENCE ON EPIDEMIOLOGY OF THE DISEASE

Continued periods of wet weather favor the development of an epidemic of black chaff. Salmon and Throckmorton (18) reported that the disease is often prevalent in Kansas in wet seasons. John-

ston<sup>9</sup> also reported that wet weather favored the disease. These reports are in agreement with observations made by the writer, particularly when the relative humidity is taken into consideration. In 1928 and 1929, particularly in 1928, there were fairly severe epidemics of the disease at University Farm, while during the seasons 1930, 1931, and 1932 there were unusually light infections. Since black chaff does not usually appear there until wheat is approaching maturity, only the weather conditions existing during June and July will be discussed in relation to the development of the disease. During these 2 months in 1930, 1931, and 1932 the weather was extremely hot and dry, while in 1928 and 1929 it more nearly approached normal. In each of the 3 years in which there was light infection the total precipitation was less and the mean monthly temperature was higher for June and July than in the 2 years when black chaff infection was heavy. The relative humidity throughout the maturative period probably had a greater influence on the development of an epidemic of black chaff than the total precipitation and mean temperature.

TABLE 5.—Mean relative humidities (separately and combined) for June and July, 1928 to 1932, inclusive, at St. Paul, Minn.

Year	Mean relative humidity											
	June				July				Seasonal average			
	7 a. m.	Noon	7 p. m.	Average	7 a. m.	Noon	7 p. m.	Average	7 a. m.	Noon	7 p. m.	Average
	Percent	Percent	Percent	Percent	Percent	Percent	Percent	Percent	Percent	Percent	Percent	Percent
1928.....	75.0	60.0	52.0	62.3	81.9	60.2	54.5	65.5	78.5	60.1	52.3	63.6
1929.....	81.0	60.6	47.6	63.1	76.2	58.0	49.1	61.1	78.6	59.3	48.4	62.1
1930.....	75.9	52.4	51.9	60.1	76.0	46.2	48.0	56.7	76.0	49.3	50.0	58.4
1931.....	77.6	53.0	53.7	61.4	68.3	39.1	37.9	48.4	73.0	46.1	45.8	55.0
1932.....	70.0	50.0	48.2	56.1	75.4	49.2	44.7	56.4	72.7	49.6	52.3	58.2

Table 5 gives the percentage of the mean relative humidity at three different hours of the day for June and July from 1928 to 1932. Apparently there is an association of high average relative humidity with heavy black chaff infection. This association is more evident if only the 2 years 1928 and 1931 are compared. The records show that at University Farm the heaviest infections in the 5-year period were in 1928 and the lightest in 1931. During June there was only a slight difference in the average relative humidity in the 2 years, but in July there were differences of 13.6, 21.1, and 16.6 percent in the 7 a. m., 12 m., and 7 p. m. readings, respectively. The close association between high relative humidity and heavy infection and lower relative humidity and less infection indicates that the existing relative humidity does determine to some extent the amount of the disease that may develop in a given season.

#### GRAINS AND GRASSES AS HOSTS

Jones, Johnson, and Reddy (13) state that the organism causing bacterial blight of wheat was pathogenic on wheat, barley (*Hordeum vulgare* L.), rye (*Secale cereale* L.), and spelt (*Triticum spelta* L.).

<sup>9</sup> JOHNSTON, C. O. See footnote 8.

No wider host range has since been reported, as far as can be ascertained, except that given earlier by the writer (1).

The writer has also obtained typical infection on einkorn (*Triticum monococcum* L.) in the field by hypodermic inoculation and on Victory oats (*Avena sativa* L.), both in the greenhouse on seedlings and in the field on adult plants. A number of other varieties of oats were inoculated in the field, but typical translucent striations developed only on Victory and Kanota. Some of the other varieties appeared to be infected soon after inoculation, but as the plants grew older the lesions became less distinct. Other workers have inoculated oats with *Bacterium translucens* var. *undulosum* but obtained no infection (13). Their failure to get infection might have been due to the fact that either the varieties of oats they used were resistant to *Bact. translucens* var. *undulosum* or the strain of the organism used as inoculum was not pathogenic on oats.

Wild grasses may also become infected with *Bacterium translucens* var. *undulosum*. The following species of grasses were hypodermically inoculated with culture M: *Bromus inermis* Leyss., *B. tectorum* L., *Hordeum jubatum* L., *Setaria lutescens* (Weigel) F. T. Hubb., *Agropyron repens* (L.) Beauv., *Dactylis glomerata* L., *Alopecurus geniculatus* L., and *Phalaris arundinacea* L. On *B. inermis* and *H. jubatum* irregular water-soaked striations appeared in 8 to 12 days. These became darker after a few days and were very similar to the lesions on the leaves of wheat. The other grasses did not become infected or, if infected, the lesions were not typical for black chaff on wheat.

#### VARIETAL SUSCEPTIBILITY IN WHEAT

All varieties of wheat are apparently more or less susceptible to the disease. At least a trace of infection can be found on field plots of most varieties of wheat in years in which there is an abundance of black chaff. Every variety tested by experimental inoculations has been susceptible to some degree. Goulden and Neatby (?) found in 1928 that practically every variety and strain in the nursery at Winnipeg, Canada, was attacked in some degree. At University Farm (Minnesota), the relative susceptibility of some of the more common varieties grown in the field and naturally inoculated was as follows: Hope, H-44, and Kota, very susceptible; Marquillo and Ceres, moderately susceptible; Marquis, Mindum, and Kubanka, resistant. Under other environmental conditions this comparative classification might not hold. Burton (4, Rept. 1931) reported that in a comparative yield test of wheat varieties carried out in 1931 at the Njaro Plant Breeding Station, Kenya, all the plants of every variety used were destroyed by black chaff, the grain being so shriveled that it could not be threshed. This report tends to bear out the conclusion of the writer that resistance to the disease is only relative.

Fifty varieties and hybrid selections were hypodermically inoculated in the booting stage with four different cultures of pathogenic bacteria. The differences noted in the virulence of the different strains of the bacteria on the varieties of wheat are indicated in the following way: 0 represents absence of the disease, and 1 to 4 represent the relative degrees of infection in any particular plot as compared with that in other plots. Table 6 gives the comparative reaction of 20 of the varieties to the four pathogens.

TABLE 6—Susceptibility of varieties of wheat to three cultures (W-1, W-2, and M-1) of *Bacterium translucens* var. *undulosum* from wheat and to one culture (B-1) from barley

Variety or nursery no.	Minn no.	C. I. <sup>1</sup> no.	Relative susceptibility <sup>2</sup> to—				Variety or nursery no.	Minn no.	C. I. <sup>1</sup> no.	Relative susceptibility <sup>2</sup> to—			
			W-1	W-2	M-1	B-1				W-1	W-2	M-1	B-1
Marquis.....	1239	3641	3	3	2	3	Mindum.....	470	5296	3	2	3	2
Marquillo.....	2202	6837	2	1	1	2	Kubanka.....	2310	1440	3	3	1	3
Ceres.....	2223	6900	3	3	1	3	Minor.....	2612	-----	3	4	3	---
Kota.....	2151	5873	3	3	3	3	Komar.....	2214	8004	2	3	4	1
Hope.....	2297	8178	3	3	1	3	II-21-7.....	2302	10002	3	3	1	3
H-44.....	2301	8177	3	2	2	2	II-21-28.....	2303	10003	2	3	0	3
Reward.....	2204	8182	2	2	1	2	II-21-48.....	2315	10020	3	3	1	2
Reliance.....	2308	7370	4	4	1	3	II-21-84.....	2316	10021	4	2	1	3
Supreme.....	2309	8026	3	3	2	2	II-21-86.....	2607	11485	3	2	3	2
Haynes Blue-stem.....	169	2874	3	3	2	3	II-21-94.....	2340	11487	3	2	1	3

<sup>1</sup> Accession number of the Division of Cereal Crops and Diseases, formerly Office of Cereal Investigations.<sup>2</sup> The numbers 0 to 4 designate the relative susceptibility of the varieties, 0 indicating absence of the disease and 4 the presence of large infected areas.

These results indicate that certain varieties may be rather resistant to some strains of the black chaff organism but quite susceptible to others. They also show that varieties like Marquis, Mindum, and Kubanka, which appear resistant in the field, may become rather heavily infected when hypodermically inoculated. The exact cause of the change in reaction to the pathogen in certain varieties is not known, but there is at least one possible explanation. Resistance to black chaff under natural conditions may be morphological or functional, as it apparently is in some cases of resistance of wheats to stem rust (9), and this resistance may be broken down when hypodermic inoculations are made.

#### COMPARISON OF PATHOGENICITY OF BACTERIUM TRANSLUCENS VAR. UNDULOSUM WITH THAT OF ISOLATIONS FROM NATURALLY INFECTED BARLEY AND RYE

In 1931 a number of varieties and selfed lines of rye at University Farm were heavily infected with a bacterial disease with symptoms closely resembling those described by Reddy, Godkin, and Johnson (17) for bacterial blight of rye (*Bacterium translucens* var. *secalis*). In the same year Manchuria barley was also found to be infected with a bacterial disease similar to that described by Jones, Johnson, and Reddy (14) as bacterial blight (*Bact. translucens*). Repeated isolations from the infected rye and barley gave yellow, slime-producing bacteria agreeing with the description of *Bact. translucens*. Marquis and Marquillo wheat inoculated with the cultures isolated from barley readily became infected. The type of lesion produced on these hosts was indistinguishable from that produced by the wheat organism. Hope wheat inoculated with cultures isolated from the infected rye was as severely attacked as when inoculated with *Bact. translucens* var. *undulosum*.

In 1932 small focal infections appeared on the first or second leaves of seedlings of several varieties and hybrid selections of barley. Cultures of bacteria isolated from these lesions were used to inoculate wheat and barley, and the same varieties were inoculated with

*Bacterium translucens* var. *undulosum* from wheat. In these tests the organism from barley was more virulent on barley than on wheat, and the organism from wheat was more virulent on wheat than on barley. However, from the results reported in table 6, in which the pathogenicity of one of the cultures (B-1) of the organism from barley was compared with that of cultures W-1, W-2, and M-1 of *Bact. translucens* var. *undulosum* from wheat on 20 varieties of wheat, it seems that the organism from barley is about as virulent on wheat as cultures W-1 and W-2 from wheat, and in most cases more virulent than culture M-1 from wheat, at least when experimentally inoculated into these wheat varieties.

#### DISCUSSION AND CONCLUSIONS

*Bacterium translucens* var. *undulosum* apparently is a group name including a number of strains of bacteria pathogenic to wheat. These strains may differ from each other in one or more of the following characters: (1) Color of colonies, (2) amount and fluidity of slime produced, (3) action on sugars, and (4) pathogenicity. Even among the limited number of isolates with which the writer worked, considerable differences were found with respect to the first three characters listed. These differences appeared to be as great as or greater than the differences heretofore used to distinguish the three similar organisms, *Bact. translucens*, *Bact. translucens* var. *undulosum*, and *Bact. translucens* var. *secalis*. If there are variations within a single so-called variety as great as or greater than the differences used to distinguish the variety from other varieties, it would appear that the validity of this variety is questionable. Since such variations in cultural reactions do occur, the only basis left for distinguishing the variety is difference in pathogenicity. The question might well be raised as to how great these differences actually are.

Reddy, Godkin, and Johnson (17) summarized the pathogenic capabilities of the three organisms as follows:

*Bacterium translucens* infects only barley.

*Bact. translucens* var. *undulosum* infects wheat, barley, rye, and spelt.

*Bact. translucens* var. *secalis* infects only rye.

However, the writer found that isolates from barley and rye readily infected wheat and that the cultures of *Bacterium translucens* var. *undulosum* infected varieties of wheat, barley, rye, oats, and certain wild grasses. If it is assumed that the cultures obtained from barley and rye were *Bact. translucens* and *Bact. translucens* var. *secalis*, respectively, then no sharp difference in pathogenicity of the organisms exists. The objection may be raised, however, that the infection found on barley and rye could have been caused by *Bact. translucens* var. *undulosum*, and it would have been expected that the isolates would infect wheat. Since pathogenicity is apparently the only valid criterion for distinguishing the three organisms, it is impossible to prove that the isolates were not the wheat organism. On the other hand, if these did belong to the variety *undulosum* wide variations evidently exist within this variety. A large number of varieties of barley and rye were infected in the plots from which the isolates were obtained. If the variety *undulosum* can be found so frequently on barley and rye, then it would be difficult to determine, without making extensive cross-inoculation tests, which

organism is involved when infection is found on these hosts. But this is evidently very impracticable, and, since a number of different pathogenic strains do exist in the variety *undulosum* and probably in the other varieties of the organism too, the most logical solution to the problem of the identity of the organisms causing bacterial blight of barley and rye and black chaff of wheat would seem to the writer to be to include all the strains under a single name. He does not believe that the differences in cultural characters are great enough or consistent enough to be used as a basis for separation, nor that the pathogenic capabilities are specific enough to warrant the separation of the group into three different varieties.

The inconsistency of the present nomenclature of this group of pathogens is evident to anyone who has worked with them. Obviously, *Bacterium translucens* should be given a varietal name if it is to be used, as it is now, to designate a variety comparable with the varieties *undulosum* and *secalis*. But within *Bact. translucens* var. *undulosum* there are evidently pathogenic strains capable of infecting a greater or less number of varieties of wheat, barley, rye, oats, and wild grasses. A similar condition may be found among the strains that make up the varieties that are supposed to be restricted to barley and rye. More work must be done to determine the extent of specialization within the entire group. It seems to the writer that it would be best to use the name *Bact. translucens* for all the strains, recognizing that it is a group species comprising a number of pathogenic strains more or less specialized as to host range. Whether the strains could properly be grouped into varieties seems questionable at present.

The symptoms of black chaff have been shown to vary with different varieties and different environmental conditions. Although the disease is usually characterized by the dark striate or streak lesions on the glumes, awns, and upper parts of the culms, it may attack all parts of the plants. Goulden and Neatby (7) called attention to two different types of bacterial disease on different varieties in the wheat nursery at Winnipeg, Canada, in 1928, and suggested that there was a possibility of two organisms being responsible. In 1931, the writer examined a number of specimens of Marquillo and Ceres wheat collected at Hallock, Minn. These were infected with a bacterial disease, the appearance of which was not typical of that usually associated with black chaff. The infected areas were somewhat oily in appearance, making the surfaces seem glossy or polished. Black chaff lesions are usually dull brown or black. There are several possible explanations of the differences noted in the appearance of the disease: (1) Different strains of *Bacterium translucens* var. *undulosum* may be responsible; (2) bacteria of entirely different species may cause a similar disease; or (3) variations in environment may cause different symptoms on different hosts. Much more extensive work must be done before the exact cause or causes of the differences in symptoms can be given.

Just how and where the black chaff organism overwinters have not been definitely shown. Seed transmission evidently plays a part in the dissemination of the disease in the soft red winter wheat area. From the results of experiments the writer concludes that the pathogen may overwinter in Minnesota in the soil or on diseased stubble and refuse. Since the organism withstood long periods of subfreezing

temperatures and also remained viable after being subjected to temperatures as low as  $-33.3^{\circ}\text{C.}$ , it seems unlikely that low temperatures would prevent its overwintering in wheat fields in this area. Also, since the changing temperature and alternate freezing and thawing during the winter and spring did not destroy the viability of the organism in soil cultures, these conditions are probably not lethal to the organism in the field. Therefore, it seems very probable that *Bacterium translucens* var. *undulosum* does withstand the adversities of winter in wheat fields and is present to infect grain the following year. Seed transmission evidently plays a smaller part in carrying the disease over from one year to the next in the hard red spring wheat area than in some other regions, so the presence of overwintered bacteria in the soil may be one of the major factors in the incidence of the disease.

Environment limits to a great extent the development of the organism and the occurrence of the disease. In general, the same conditions favor both the growth of the organism and the development of epidemics. The best growth of the organism in culture occurs at relatively high temperatures and high relative humidities, and the most severe epidemics of the disease occur in warm, wet seasons. However, low relative humidity is probably more often a limiting factor in the development of the disease than is low temperature. Apparently, low temperatures merely retard the advance of the organism through the host tissues during cool weather without preventing its rapid development again as soon as the temperature rises. As a result the disease may advance rapidly if the days are warm, even though the nights are cool. On the other hand, the advance of the organism through the tissues already attacked may be checked for some time by the effect of low humidity drying out the lesions. The tissues of cereal plants are not bulky and are protected from desiccation only by the cuticle and the action of the guard cells in the closing of the stomata. In the infected areas the guard cells are inactivated by the attack of the pathogen and the tissues in the lesions are exposed to desiccation. Cells adjacent to those already parasitized probably are also dried out and killed, thus preventing further extension of the lesions even when more humid conditions exist later. Hence, it seems reasonable to conclude that short periods of low relative humidity have a more lasting detrimental effect on the development of the disease than periods of low temperature. As a result, one might reason that seasons in which the temperature was comparatively low and the relative humidity high would be more favorable for the appearance of an epidemic of black chaff than seasons with high temperatures and low relative humidity.

#### SUMMARY

Black chaff of wheat, caused by *Bacterium translucens* var. *undulosum*, appears to be very widely distributed. It occurs in the United States, Canada, and Mexico, in North America, and in Russia, France, and Belgium, in Europe. It also is found in Asia and Africa.

All aboveground parts of susceptible plants may be attacked: Leaves, glumes, awns, rachises, and culms. Some varieties are often attacked at the lower nodes of the culms, while in others the infection is usually limited to the heads and the necks of the culms.



The channels of entrance into the host are through the stomata and wounds, and the attacks are intercellular and intracellular.

The optimum temperature for the growth of the organism in culture is between 25° and 30° C, the maximum approximately 40°, and the minimum slightly below 10°.

Low relative humidity of the surrounding atmosphere retarded the growth of the organism in culture. The retarding effect was noticeable at a relative humidity of 50 percent and was greater as the percentage was lowered.

Temperature had a marked effect on the length of incubation period required. At 20° C. from 2 to 7 days were necessary for incubation, while at 10° from 8 to 20 days were required.

Low temperatures also limited the size of the lesions caused by *Bacterium translucens* var. *undulosum*. In 30 days the organism caused only very small lesions, 1 to 5 mm in length, at 10° C, while at 20° it caused much larger lesions, i. e., 10 to 30 mm in length.

The organism is resistant to extremely low temperatures, remaining viable outside in soil cultures for at least 124 days after December 15.

The disease usually does not appear in Minnesota until wheat is headed out and approaching maturity. Seedling infection rarely occurs there.

The host range of *Bacterium translucens* var. *undulosum* was found to include oats, einkorn, *Hordeum jubatum*, and *Bromus inermis* in addition to the previously described hosts—wheat, barley, rye, and spelt.

There is considerable variation in susceptibility of varieties of wheat to black chaff. Field observations over a number of years indicate that Hope, H-44, and Kota are very susceptible; Marquillo and Ceres moderately susceptible; and Marquis, Mindum, and Kubanka resistant.

Strains of the organism exist which differ in cultural and physiological characters and in pathogenicity.

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# THREE BLUE-STAINING FUNGI, INCLUDING TWO NEW SPECIES, ASSOCIATED WITH BARK BEETLES<sup>1</sup>

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## INTRODUCTION

In a previous paper<sup>4</sup> the writer described the association of the blue-stain fungus, *Ceratostomella pini* Münch, with the bark beetle, *Dendroctonus frontalis* Zimm., on the Atlantic coast and with *D. brevicornis* Lec. on the Pacific coast, as well as the association on the Atlantic coast of *C. ips* Rumbold with *Ips calligraphus* Germ., *I. grandicollis* Eichh., *I. avulsus* Eichh., and *I. pini* Say. Since the publication of the description of *C. ips*, in 1931, collections have been made of the fungus in all the Gulf States except Texas, and in Tennessee, Ohio, Wisconsin, and Minnesota. In the East, *C. ips* has been isolated from *Pinus banksiana* Lamb., *P. caribaea* Mor., and *P. taeda* L., in addition to many of the pines listed in the previously mentioned paper. It has also been isolated from adult *I. calligraphus*, *I. grandicollis*, and *I. avulsus*. The work here reported reveals the association in the West of *C. ips* with the bark beetles *I. oregoni* Eichh., *I. emarginatus* Lec., infesting *P. ponderosa* Dougl., and *I. integer* Eichh., infesting *P. ponderosa* and *Larix occidentalis* Nutt.

Two species of *Ceratostomella*, hitherto not described, were found, one associated with the beetle *Dendroctonus pseudotsugae* Hopk., which kills Douglas fir and infests western larch, and the other with *D. piceaperda* Hopk., which infests spruce in eastern Canada. Further it was found that the insects carried with them, besides the blue-stain fungi, two types of yeast—Zygosaccharomycetes and an anascosporeogenous mycelium-forming group.<sup>5</sup>

## METHODS

With one exception, the specimens described in this paper were collected by entomologists, who started the cultures of the fungi by dropping beetles cut out of the trees just as they were ready to emerge from their galleries or just after they had made successful attacks, into test tubes containing slanted malt agar.<sup>6</sup> The tubes were mailed to the laboratory, where the insects were removed. Fungi

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<sup>2</sup> The writer wishes to express thanks to the members of the Division of Forest Insects, Bureau of Entomology and Plant Quarantine, U. S. Department of Agriculture, for their collaboration, and to R. E. Balch, Division of Forest Insects, Entomological Branch, Department of Agriculture, Canada, for his cooperation.

<sup>3</sup> In cooperation with the Forest Products Laboratory, Forest Service, U. S. Department of Agriculture.  
<sup>4</sup> RUMBOLD, C. T. TWO BLUE-STAINING FUNGI ASSOCIATED WITH BARK-BEETLE INFESTATION OF PINES. Jour. Agr. Research 43, 847-873, illus. 1931.

<sup>5</sup> A study of these organisms is being made by Eugene C. Holst, student assistant, Division of Forest Pathology, Bureau of Plant Industry, U. S. Department of Agriculture.

<sup>6</sup> RUMBOLD, C. T. See footnote 4.

appearing regularly in the tubes were assumed to have started from spores on or in the bodies of the beetles. Cultures were also obtained in the laboratory by transferring frass, slivers of the gallery walls, or adjacent stained wood to malt agar. Cultures from the wood were compared with the cultures obtained from the beetles. A few larvae and eggs cut out of the galleries also were used. The entomologists examined the material to make sure that insects of other species than the one reported were not present.

Except when otherwise stated, the material used for description and measurement was taken from cultures grown on malt agar. The cultures were kept where the temperature ranged from 11° to 30° C. with a usual temperature of 24°.

### CERATOSTOMELLA IPS

The blue-stain fungus, *Ceratostomella ips*, has already been described,<sup>7</sup> but certain additional facts about the species in general are given and certain necessary corrections are made in the present paper. For the western strains, listed in table 1, only the descriptions and measurements that differ from those of the eastern and Japanese strains are given.

TABLE 1.—Source of western strains of *Ceratostomella ips*

Date collected	Locality	Species of tree	Species of beetle	Infestation period (days)	Remarks
July 1930...	Northport, Wash....	<i>Pinus ponderosa</i> .	<i>Ips oregoni</i> .	Unknown.	Tree killed in recent fire. Blue-stained wood used as inocula.
September 1930.	Klamath Falls, Oreg.....	.....do.....	.....do.....	14.....	Inocula were interior blue-stained wood.
	(Sisters, Oreg.....	.....do.....	<i>I. integer</i> .....	2.....	Inocula were tissues from gallery wall and frass.
	.....do.....	.....do.....	.....do.....	21.....	Inocula were tissues from gallery walls.
	.....do.....	<i>Larix occidentalis</i> .	.....do.....	21.....	Green windfall. Inocula were tissues of entrance and gallery walls and interior stained wood.
June 1932.	.....do.....	<i>Pinus ponderosa</i> .	<i>I. emarginatus</i> .	14.....	Standing tree with fading needles. Inocula were gallery walls.
	.....do.....	.....do.....	.....do.....	21.....	Cultures started from larval mines cut from green windfall.
	Coeur d'Alene, Idaho.....	.....do.....	<i>I. oregoni</i> .....	About 65.	Inocula were emerging adult beetles cut from standing tree.
	(Sisters, Oreg.....	.....do.....	<i>I. integer</i> .....	About 21.	Inocula were adult beetles cut from a green windfall.
June 1933.	.....do.....	.....do.....	.....do.....	21.....	Green windfall. Perithecia in the galleries. Gallery walls used as inocula.
	.....do.....	.....do.....	<i>I. emarginatus</i> .	Unknown.	Adult beetles cut from standing tree used as inocula.
	Coeur d'Alene, Idaho.....	.....do.....	<i>I. oregoni</i> .....	About 7...	Do.

### THE FUNGUS IN IPS-INFESTED BARK AND WOOD

The appearance of *Ceratostomella ips* in infested bark and wood in the West is typical of that found in the East. Additional information obtained in the West showed that the perithecia of *C. ips* lined

<sup>7</sup> RUMBOLD, C. T. See footnote 4.

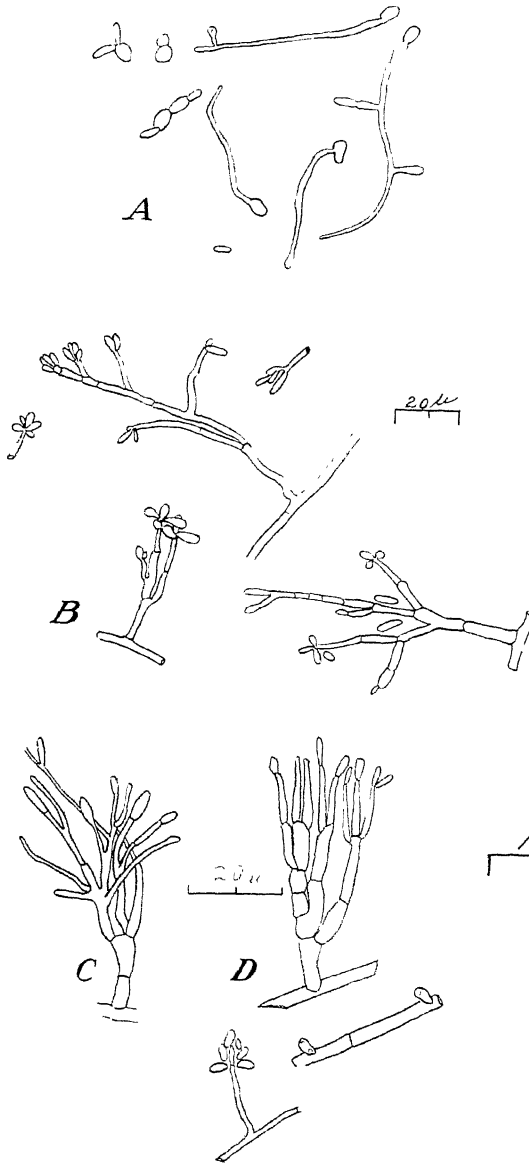


FIGURE 1.—Camera-lucida drawings of *Ceratostomella ips* growing in Van Tieghem cells on malt agar. A and B, Strain isolated from adult *Ips integer* collected in Oregon. A, Germinating conidia in a 1-day-old culture; B, conidia and conidiophores when culture was 3 days old; C, strain isolated from adult *I. oregoni* collected in Idaho; conidia and conidiophores in a 2-day-old culture; D, strain isolated from adult *I. emarginatus* collected in Oregon, mycelium, conidia, and conidiophores in a 2-week-old culture.



FIGURE 2.—A fascicle of mature conidiophores of *Ceratostomella ips* associated with *Ips integer*.

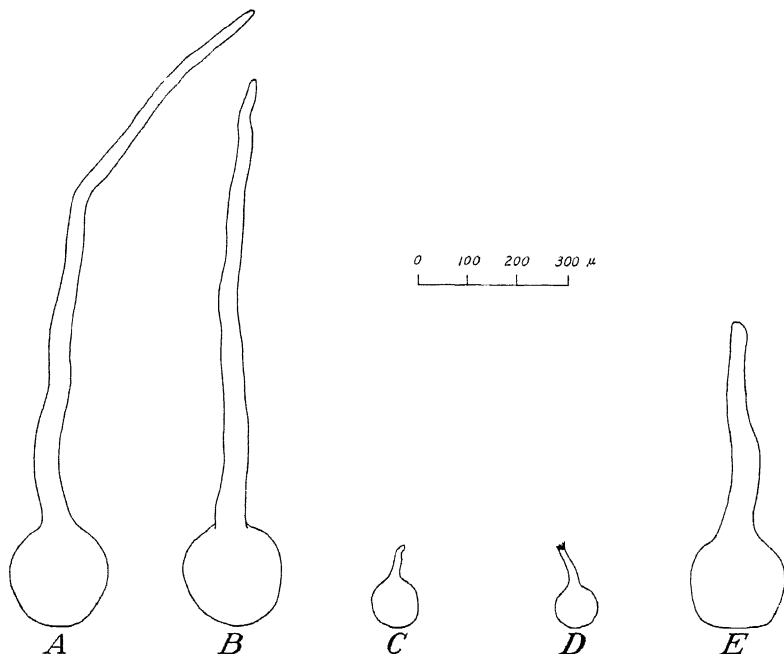


FIGURE 3.—Outlines of perithecia drawn according to average dimensions: A, *Ceratostomella ips* associated with eastern species of *Ips*; B, *C. ips* associated with western species of *Ips*; C, *C. pini* associated with some eastern and western species of *Dendroctonus*; D, *C. pseudotsugae* associated with *D. pseudotsugae*; E, *C. piceaperda* associated with *D. piceaperda*.

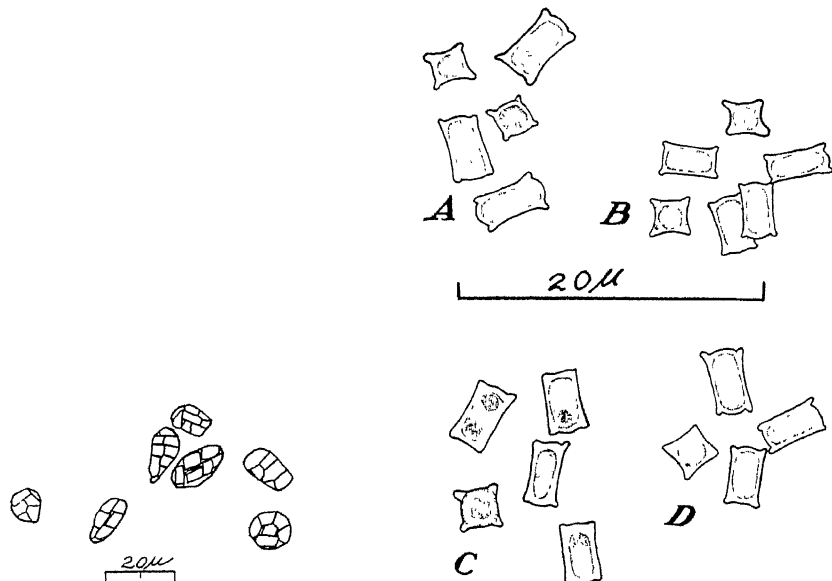


FIGURE 4.—Asci of a strain of *Ceratostomella ips* associated with *Ips emarginatus*.

FIGURE 5.—Ascospores of *Ceratostomella ips* associated with (A) *Ips emarginatus*, (B) *I. oregoni*, (C) *I. integer*, and (D) *I. calligraphus*.

TABLE 2.—Summary of mean dimensions of *Ceratostomella ips* from eastern and western strains grown on malt agar

Associated insect	Source		Strains of <i>Ceratostomella ips</i> having perithecia with a mean dimension of—										Perithecia of <i>Ceratostomella ips</i> having a mean dimension of—									
			Grand mean																			
			Host	Region	150 $\mu$	170 $\mu$	190 $\mu$	210 $\mu$	230 $\mu$	250 $\mu$	270 $\mu$	290 $\mu$	150 $\mu$	170 $\mu$	190 $\mu$	210 $\mu$	230 $\mu$	250 $\mu$	270 $\mu$	290 $\mu$		
<i>Ips</i> spp.-----	2- to 3-needled pines.	Atlantic States. <sup>2</sup>	Num-ber 1	Num-ber 1	Num-ber 3	Num-ber 7	Num-ber 2	Num-ber 1	Num-ber 1	Num-ber 1	206	Num-ber 10	Num-ber 67	Num-ber 74	Num-ber 284	Num-ber 99	Num-ber 10	Num-ber 7	Num-ber 31			
<i>I. integer</i> -----	<i>Pinus ponderosa</i>	Oregon	1	1	6	5	1	1	1	1	198	10	227	227	284	99	10	7	31			
Do.-----	<i>Larix occidentalis</i>	do.	1	1	1	1	1	1	1	1	192	52	50	50	3	47	31	20				
<i>I. emarginatus</i> -----	<i>P. ponderosa</i>	do.	1	1	1	1	1	1	1	1	236	52	52	50	22	3	47	31				
do	do	{ Oregon Washington Idaho.	2	1	1	1	1	1	1	1	191	52	50	50	22	3	47	31				
Total-----												19	171	401	405	83	54	31				
WIDTH OF BASE																						
<i>Ips</i> spp.-----	2- to 3-needled pines.	Atlantic States. <sup>4</sup>	1	3	2	8	4	1			198	19	110	95	290							
<i>I. integer</i> -----	<i>P. ponderosa</i>	Oregon	1	3	8	4	1				197	19	110	249	87							
Do.-----	<i>L. occidentalis</i>	do.			1	1	1	1	1	1	195			50	50							
<i>I. emarginatus</i> -----	<i>P. ponderosa</i>	do.			1	1	1	1	1	1	238			52	52							
do	do	{ Oregon Washington Idaho.		2	1	1	1				187			52	50	22						
Total-----												19	162	496	399	10	47	31				



## LENGTH OF NECK

			Straus of <i>Ceratostomella ips</i> having perithecia with a mean dimension of—								Grand mean	Perithecia of <i>Ceratostomella ips</i> having a mean dimension of—							
			600 $\mu$	800 $\mu$	1,000 $\mu$	1,200 $\mu$	1,400 $\mu$	1,600 $\mu$	1,800 $\mu$	2,000 $\mu$		600 $\mu$	800 $\mu$	1,000 $\mu$	1,200 $\mu$	1,400 $\mu$	1,600 $\mu$	1,800 $\mu$	2,000 $\mu$
			Num- ber	Num- ber	Num- ber	Num- ber	Num- ber	Num- ber	Num- ber	Num- ber		Num- ber	Num- ber	Num- ber	Num- ber	Num- ber	Num- ber	Num- ber	Num- ber
<i>Ips</i> spp. ....	2- to 3-needled pines.	Atlantic States, <sup>2</sup>																	
<i>I. infector</i> .....	<i>P. ponderosa</i> .....	Oregon .....	1	2	2	2	3	2	2	2	1,273	19	81	51	87	159	61	56	
Do. ....	<i>I. occidentalis</i> .....	do. ....		9	4						849	255	88						
<i>I. emarginatus</i> .....	<i>P. ponderosa</i> .....	do. ....	1	1	3						664	50		98					
<i>I. Oregon</i> .....	do. ....	Oregon .....	1	1		1	1				968	3	52						
		Washington .....									1,209	29	23		50	22			
		Idaho .....																	
Total .....												101	411	237	137	181	61	56	0

: The round numbers (microns) of the column headings are the midpoints of the groups into which the actual means fall

: Previously reported in more detail (see footnote 4).

The asci (fig. 4) resemble those found in the eastern strain but differ from the Japanese strain in that the ascospores fill the asci much more than is reported by Nisikado and Yamauti.<sup>11</sup>

When pressed out of the perithecia the ascospores of eastern and western strains have the shape of quadrangular prisms (fig. 5), not cylinders as was stated in the technical description of the eastern strains of this fungus in 1931.<sup>12</sup> The shape of the ascospores may be seen more readily if stained with picro-aniline blue. So treated, the walls of the spores become yellow and the interior blue. The edges of the walls of the quadrangular-prism-shaped spores look like flanges, while the blue-stained interiors are ellipsoidal or cylindrical. Figure 5, A, B, and C, shows the side and end views of spores of three western strains of *C. ips*, and, for comparison, spores of an eastern strain, isolated from a specimen of *Ips calligraphus*, are shown in D.

#### CERATOSTOMELLA PSEUDOTSUGAE N. SP.

*Ceratostomella pseudotsugae* n. sp. was collected during three summers on specimens of the species listed in table 3.

#### THE FUNGUS IN BARK AND WOOD INFESTED WITH DENDROCTONUS PSEUDOTSUGAE

The Douglas fir beetles, *Dendroctonus pseudotsugae*, bore through the outer bark to the phloem or to the outer layer of wood and there extend their galleries. *Ceratostomella pseudotsugae* grows from these galleries, usually in longitudinal streaks (fig. 6). The fungus covers the inner bark and wood with a black mycelial growth, which is usually mixed with particles of dried rosin. The stain of the inner bark caused by this fungus is not so noticeable as that caused by *C. pini*, possibly because the numerous black sclerotia so characteristic of the latter are not present. The fungus grows in toward the heartwood, staining the sapwood gull gray to slate gray.<sup>13</sup> The medullary rays become black.

TABLE 3.—Source of *Ceratostomella pseudotsugae* n. sp. associated with *Dendroctonus pseudotsugae*

Date collected	Locality	Species of tree	Infestation period	Remarks
September 1931..	Metalline Falls, Wash.	<i>Pseudotsuga taxifolia</i>	Days 39	Specimen showed living larvae. Sporulating perithecia growing in gallery walls
	.....do.....	.....do.....	38	Do
	.....do.....	.....do.....	35	Specimen showed living beetles and larvae. Conidiophores in the larval mines
	.....do.....	.....do.....	(1)	Attack by beetles began in May 1931. Adult beetles cut out of bark as they were emerging. 4 larvae cut out of their mines. 23 of the 24 cultures started developed perithecia.
June 1932.....	.....do.....	.....do.....	4	Inner bark tissues from entrance chamber used as inocula
	.....do.....	.....do.....	10	Eggs cut from galleries used as inocula
	.....do.....	<i>Larix occidentalis</i>	7	Large tree felled May 20, 1932. Fruss used as inocula.
June 1933.....	Sisters, Oreg....	<i>Pseudotsuga taxifolia</i>	14	A green windfall of large size. Bark tissue from gallery used as inocula.
	Metalline Falls, Wash.	.....do.....	(1)	Beetles cut from bark as they were emerging. 10 out of 11 cultures started developed perithecia.

<sup>1</sup> Unknown.

<sup>11</sup> NISIKADO, Y., and YAMAUTI, K. CONTRIBUTIONS TO THE KNOWLEDGE OF THE SAP STAINS OF WOOD IN JAPAN. I. STUDIES ON CERATOSTOMELLA IPS RUMBOLD, THE CAUSE OF A BLUE STAIN OF PINE TREES IN WESTERN JAPAN. Ber. Ohara Inst. Landw. Forsch. 5: 501-538, illus. 1933.

<sup>12</sup> RUMBOLD, C. T. See footnote 4.

<sup>13</sup> RIDGWAY, R. COLOR STANDARDS AND COLOR NOMENCLATURE. 43 pp., illus. Washington, D. C. 1912.



FIGURE 6.—Inner bark of *Pseudotsuga taxifolia* (LaMarck) Britton showing galleries of *Dendroctonus pseudotsugae*. Blue stain caused by *Ceratostomella pseudotsugae* extends from the galleries. The tiny black dots are perithecia.

On the black mycelial growth are tiny perithecia (fig. 3, *D*) with short curved necks. The combination of beetle galleries and spreading fungus loosens the bark. In a short time ants, mites, nematodes, and various molds are found under the bark.

*Ceratostomella pseudotsugae* first invades the ray-parenchyma cells of the wood and later the tracheids, passing from cell to cell through the pits.

#### THE FUNGUS IN CULTURE

Cultures of *Ceratostomella pseudotsugae* were started from adult beetles, from slivers of the insect gallery walls, and from frass, eggs, and larvae. All these cultures developed yeast at first, and later the blue-stain fungus grew out from the yeast colonies, often covering them completely.

In culture this fungus develops a large amount of aerial mycelium and produces a lighter-colored mycelium, more conidia, and fewer perithecia than those developed in *Ceratostomella pini* associated with *Dendroctonus brevicomis* and *D. frontalis*. In this respect it resembles somewhat a strain of *C. pini* found in the East on *Pinus virginiana* Mill., unassociated with *Dendroctonus*,<sup>14</sup> and a strain found on the Pacific coast, isolated from a lightning-injured ponderosa pine. However, the lack of sclerotia, both when grown on malt agar and on pine, distinguishes this fungus from *C. pini*, and it differs also in its physiological reactions.

*Ceratostomella pseudotsugae* and three strains of *C. pini* were grown on media having different sources of nitrogen.<sup>15</sup> To the standard basal medium (containing 2.0 percent of glucose, 0.50 percent of acid potassium phosphate, 0.1 percent of magnesium sulphate, 0.0016 percent of bromcresol purple, and 1.5 percent of Bacto-agar) was added 0.5 percent of one of the following nitrogenous compounds: Urea, asparagine, sodium caseinate, or peptone. The media were all adjusted to approximately pH 6.2. Table 4 gives the reactions. By observing the reaction changes of the media over a period of time, or the presence or absence of growth and the type of growth, it was found that the three strains of *C. pini* reacted alike, growing abnormally and producing acid when supplied with sodium caseinate or peptone but not growing on asparagine or urea; *C. pseudotsugae* grew well on all the media, producing a basic reaction on those containing sodium caseinate or peptone. These physiological reactions emphasize the distinction between *C. pseudotsugae* and *C. pini*.

TABLE 4.—Reaction of *Ceratostomella pini* and *C. pseudotsugae* on media containing different nitrogenous compounds

Culture and source	Reaction to indicated compound after specified period					
	Sodium caseinate			Peptone	Asparagine	Urea
	3 days	8 days	28 days	21 days	21 days	21 days
<i>Ceratostomella pini</i> no. 187a from <i>Dendroctonus frontalis</i> , North Carolina.	Acid....	Acid....	Acid....	Acid....	No growth.	No growth.
<i>C. pini</i> no. 416 from <i>D. brevicomis</i> , Oregon.	...do....	...do....	...do....	...do....	...do....	Do
<i>C. pini</i> no. 421 from uninfested wood, Oregon.	...do....	...do....	...do....	...do....	...do....	Do.
<i>C. pseudotsugae</i> no. 436 from <i>D. pseudotsugae</i> , Washington.	...do....	Neutral.	Basic....	Basic....	Growth....	Growth.

<sup>14</sup> RUMBOLD, C. T. See footnote 4.

<sup>15</sup> These tests were made by Eugene C. Holst.

When the fungi are grown on autoclaved pine, the difference between a strain of *Ceratostomella pini* isolated from an adult *Dendroctonus brevicornis* and the fungus isolated from a *D. pseudotsugae* beetle is easily seen. *C. pini* covers the surface of the wood quickly with a black stain consisting of innumerable perithecia, black sclerotia, and dark-colored mycelium. The wood has a black granular appearance. *C. pseudotsugae* stains the wood a gull gray, the aerial mycelium is hyaline to gray, but most of the surface discoloration is caused by the numerous perithecia.

TABLE 5—Dimensions of perithecia of *Ceratostomella pseudotsugae* from *Dendroctonus pseudotsugae* beetles or wood infested with them

GROWN ON MALT AGAR

Source and strain number of <i>Ceratostomella pseudotsugae</i>	Fruits measured	Height of base			Width of base			Length of neck		
		Ex-treme range	Sextile range <sup>1</sup>	Mean	Ex-treme range	Sextile range <sup>1</sup>	Mean	Ex-treme range	Sextile range <sup>1</sup>	Mean
	Number									
From <i>Pseudotsuga taxifolia</i> , 1931:										
No 383.....	26	60-120	80-110	95	60-120	80-110	94	40-120	60-90	74
No 384.....	91	50-130	70-100	85	50-120	70-100	85	40-110	55-96	75
From <i>Larix occidentalis</i> , 1932:										
No. 423b.....	50	70-120	80-100	90	60-130	80-110	91	30-140	50-80	67
From <i>P. taxifolia</i> , 1932:										
No 423a.....	30	45-140	70-90	81	45-140	70-100	84	20-100	60-90	76
No. 423c.....	46	50-130	80-110	90	50-120	80-110	89	30-110	50-75	60
Associated with <i>Dendroctonus pseudotsugae</i> , 1932:										
No. 423:										
Adult 1.....	63	60-102	65-80	73	55-100	64-80	74	45-125	73-110	93
Adult 2.....	20	55-90	60-80	70	55-83	65-75	70	40-100	40-63	54
Adult 3.....	31	55-100	70-92	82	50-110	70-90	79	55-160	78-130	103
Adult 9.....	36	61-100	70-95	82	42-110	70-90	81	30-130	90-115	103
Adults 7, 10, 11, 12, 13, 17.	33	50-130	60-90	80	50-120	60-90	80	45-120	60-100	80
Associated with <i>D. pseudotsugae</i> , 1933:										
No 436:										
Adults 3, 5, and 7.....	24	60-120	75-110	91	50-100	70-100	80	30-90	40-80	62
Adult 6.....	30	60-130	70-90	80	60-130	65-80	77	45-130	65-100	82
Adults 8 and 10.....	31	60-110	70-100	85	60-110	70-100	81	40-90	50-80	67
Adult 9.....	74	50-140	70-110	87	50-130	70-110	87	45-130	60-110	90
Total, range, or mean.	585	45-140	70-100	84	42-140	70-100	83	20-160	55-100	79

GROWN ON WOOD

Associated with <i>D. pseudotsugae</i> in <i>P. taxifolia</i> : No 436, adult 6, grown on <i>Pinus taeda</i> .....	40	45-100	60- 80	70	40-100	60- 80	70	30-120	50- 95	74
No. 383, original perithecia growing on inner bark of <i>Pseudotsuga taxifolia</i> .....	13	50- 90	50- 80	69	50- 80	60- 80	68	35-110	45- 90	70

<sup>1</sup> The sextile range discards the sixth of the measurements at each end of the extreme range, permitting an approximation of the frequency distribution

A mature culture of *Ceratostomella pseudotsugae* contains white gray, and snuff-brown mycelium at its top, and numerous small, often incompletely developed perithecia. Black perithecia embedded in a warm sepia and black mycelium, surrounded and sometimes covered by gray aerial mycelium, appear in the center of the slant. The individual perithecia cannot be seen easily with the naked eye but are visible with a hand lens. At the base of the slant, wedged

between the glass and agar, a black sclerotoid layer of mycelium sometimes forms. The fungus grows readily but not vigorously on steam-sterilized southern yellow pine. A culture, made in the forest from gallery-wall tissue, when 10 days old contained gray, brown, and black mycelium and mature perithecia, but transplants from this strain remained light-colored and failed to produce spores. The perithecia developed on wood are somewhat smaller than those growing on malt agar (table 5).

#### MYCELIUM

The young mycelium growing in culture forms a white mat covering the surface of the agar. The young hyaline hyphae are septate, branch irregularly, and measure  $1.2\mu$  to  $4\mu$  in diameter. They form strands, anastomose, and change color gradually. The hyphae turn brown with age and the cell divisions become numerous, so that old hyphae often consist of short cells that may become globular. The walls thicken, and often a dark granular exudation appears. Old dark-colored hyphae may be relatively thick, varying from  $4\mu$  to  $15\mu$  in diameter. The cells forming the old hyphae often become polyhedral and in this respect resemble those forming the sclerotia of *Ceratostomella pini*. However, their walls are not so thick nor so firm as sclerotial cells.

#### CONIDIA AND CONIDIOPHORES

At room temperature (about  $25^{\circ}\text{C}.$ ) the first conidia appear about 48 hours after the germination of ascospores. They grow directly from the hyphae at first and are obovoid. Later they form in clusters at the tips of simple hyaline conidiophores. As the conidiophore lengthens, the conidium is pushed to the side of the growing tip. The conidiophores remain small, are usually unbranched (fig. 7, *A*), and do not change color as the culture ages. The conidia vary in shape from globular or obovoid to clavate, and in size from  $2.7\mu$  to  $5\mu$  by  $1.4\mu$  to  $2.7\mu$ .

#### PERITHECIA

Beneath the bark of the tree the black perithecia develop on the dark mycelium, which spreads from the galleries between the inner bark and the wood. The fruits generally are flattened by the pressure of the bark. In this situation the base of the perithecium is bare of hyphae except those attaching it to the substratum. In culture perithecia are found with basal hyphae growing into the air, looking like bristles. They are straight, septate, brown, tapering at the tip, about as long as the width of the base of the perithecium. The ostiole at the end of a short curved neck is surrounded by short hyaline cilia from  $10\mu$  to  $23\mu$  long (fig. 3, *D*). Ten have been counted at an ostiole. An ostiole through which ascospores have been ejected is generally torn. The globular perithecial base is dark brown and the tip of the neck light brown.

The perithecia somewhat resemble those of *Ceratostomella pini* (fig. 3, C). The measurements of the perithecia are given in table 5.

#### Asci

So far only immature asci have been seen. These were clavate and later polyhedral, owing possibly to their being crowded in the interior of the base. The eight spores are irregularly arranged. The walls of the asci disappear before the ascospores are ejected. There

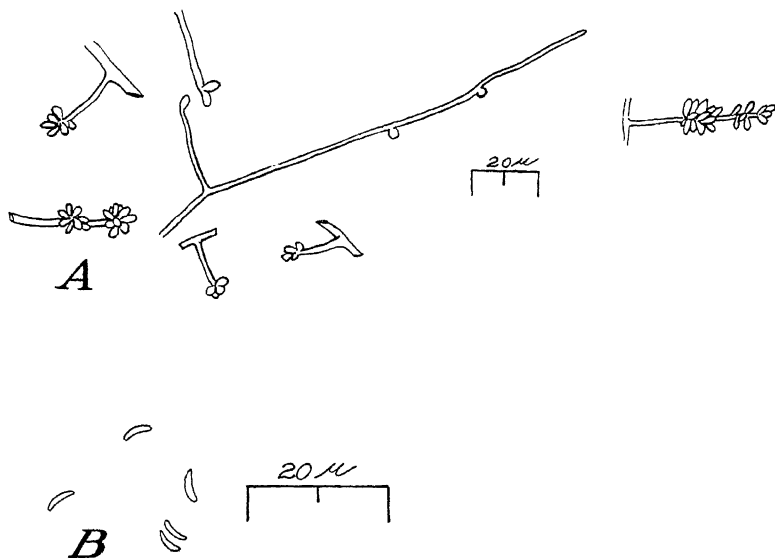


FIGURE 7.—*Ceratostomella pseudotsugae* growing in Van Tieghem cell cultures 1 to 5 days old: A, Conidia and conidiophores; B, ascospores.

is an oily substance formed with the spores that often is ejected before the spores.

#### ASCOSPORES

The ascospores are crescent-shaped, slender, and pointed at both ends (fig. 7, B). They are hyaline, with a thin mucilaginous coating which causes them to clump together when ejected on the tip of the neck of the perithecium. The glistening clumps of ascospores are conspicuous. The mean length of the ascospores is  $4\mu$ , and the mean width  $1.5\mu$ .

#### TECHNICAL DESCRIPTION

##### *Ceratostomella pseudotsugae* n. sp.

Young colonies with conidia white changing to sepia and black, with perithecia; young hyphae hyaline,  $1.2\mu$  to  $4.0\mu$  in diameter; old hyphae brown,  $4\mu$  to  $15\mu$  in diameter; conidia hyaline, appearing first on hyphae, globular, obovoid to clavate, ranging from  $2.7\mu$  to  $5\mu$  by  $1.4\mu$  to  $2.7\mu$ ; conidiophores single, hyaline, usually unbranched, hyphae bearing conidia in clusters; conidia solitary, adherent, forming a head; perithecia black, globose, slightly hirsute; height of base, extreme range  $45\mu$  to  $140\mu$ , sextile range  $70\mu$  to  $100\mu$ , mean  $84\mu$ ; width of base, extreme range  $42\mu$  to  $140\mu$ , sextile range  $70\mu$  to  $100\mu$ , mean  $83\mu$ ; length of neck, extreme range  $20\mu$  to  $160\mu$ , sextile range  $55\mu$  to  $100\mu$ , mean  $79\mu$ ; diameter of neck near base  $25\mu$ , at ostiole  $10\mu$ , the ostiole with hyaline terminal cilia  $10\mu$  to  $23\mu$  long;

asci ephemeral, polyhedral; ascospores 8, hyaline, crescent-shaped; length, extreme range  $2.4\mu$  to  $5.4\mu$ , sextile range  $3.5\mu$  to  $4.8\mu$ , mean  $4\mu$ ; width, extreme range  $0.9\mu$  to  $2.4\mu$ , sextile range  $1.2\mu$  to  $1.9\mu$ , mean width  $1.5\mu$ .

On sapwood of *Pseudotsuga taxifolia* infested with *Dendroctonus pseudotsugae* near Metaline Falls, Wash., and near Sisters, Oreg.; and on *Larix occidentalis* infested with *D. pseudotsugae*, near Metaline Falls, Wash.

#### DIAGNOSIS

#### *Ceratostomella pseudotsugae* sp. nov.

Coloniis juvenilibus conidiferis albis, tum brunneis-nigrescentibus cum peritheciis; hyphis hyalinis,  $1.2\mu$ – $4.0\mu$  diam., dein brunnescentibus,  $4\mu$ – $15\mu$  diam.; conidiophoris simplicibus, hyphis plerumque sine ramis conidia globosa ferentibus; conidiis hyalinis, in hyphis primo apparentibus, globosis, obovoideis vel clavatis, solitariis deinde in massulas congregatis,  $2.7\mu$ – $5.0\mu$  usque  $1.4\mu$ – $2.7\mu$ ; peritheciis nigris, globosis lentius hirsutis,  $45\mu$ – $140\mu$  imo altis,  $42\mu$ – $140\mu$  latis; rostellis  $20\mu$ – $160\mu$  longis,  $10\mu$ – $25\mu$  diam., ostiolo filamentis hyalinis, terminalibus,  $10\mu$ – $23\mu$  longis, praedito; ascis evanidis, polyhedricis, octosporis; ascosporis hyalinis, lunatis,  $2.4\mu$ – $5.4\mu$  longis,  $0.9\mu$ – $2.4\mu$  latis.

In ligno sapido *Pseudotsugae taxifoliae* et *Laricis occidentalis* *Dendroctonus pseudotsugae* infestato, Washington et Oregon, U. S. A.

#### CERATOSTOMELLA PICEAPERDA N. SP.

*Ceratostomella piceaperda* n. sp. was found on the following specimens collected in the localities named:

*Picea glauca* (Moench) Voss, infested 15 days with *Dendroctonus piceaperda*, collected in the forest near St. Peters, Cape Breton, Nova Scotia, Canada, in June 1930.

*Picea glauca*, infested 1 month with *Dendroctonus piceaperda*, collected in the forest near Grand Cascadepia, Bonaventure County, on the Gaspé Peninsula, Province of Quebec, Canada, in July 1931.

An adult beetle of *D. piceaperda*, in July 1933, cut out of the bark of *P. glauca*. The tree stood in a forest near St. Peters, Cape Breton, Nova Scotia, Canada, and had been recently attacked.

The blue-stain fungus isolated from these specimens resembles in many respects a German fungus, *Ceratostomella penicillata* Grosmann,<sup>16</sup> isolated from *Picea excelsa* Link infested with *Ips typographus* L. and *Pityogenes chalcographus* L. The measurements of the peritheciium of the German fungus are: Diameter of base,  $250\mu$  to  $300\mu$ ; length of neck,  $300\mu$  to  $500\mu$ . The ascospores measure  $6.5\mu$  by  $2.3\mu$ . The height of conidiophores varies from  $450\mu$  to  $600\mu$ , diameter  $7\mu$  to  $8\mu$ , conidia  $11\mu$  to  $12\mu$  by  $3\mu$  to  $3.5\mu$ . The conidia and conidiophores of the Canadian fungus are smaller. The conspicuous conidiophores of the German strain also differ from the Canadian in the shape and size of their heads.

#### THE FUNGUS IN BARK AND WOOD INFESTED WITH DENDROCTONUS PICEAPERDA

*Dendroctonus piceaperda* will invade living spruce trees, although it prefers the bark of dying windfalls and fresh stumps. It bores through the outer bark to the phloem or to the outer layer of wood and there extends its egg galleries (fig. 8). *Ceratostomella piceaperda* has been found fruiting in these galleries and growing from them, commonly in longitudinal streaks. A close examination of the blackened areas showed that the inner bark and cambium were covered with a network of dark-brown hyphae. Perithecia were seen only in the galleries.

<sup>16</sup> GROSMANN, H. ÜBER DIE SYSTEMATISCHEN BEZIEHUNGEN DER GATTUNG LEPTOGRAPHIUM LAGERBERG ET MEIN ZUR GATTUNG CERATOSTOMELLA SACC. NEBST EINIGEN BEMERKUNGEN ÜBER SCOPULARIA VENUSTA PREUSS UND HANTZACHIA PHYCOMYCES AWD. Hedwigia 72: 183–194, illus. 1932.



Spruce stained with *Ceratostomella piceaperda* is gull gray in color. The fungus grows first in the rays, turning them black, then invades the tracheids by means of the bordered pits. In culture the surface of infected wood appears furry because of the numerous perithecia and conidiophores covering it.

#### THE FUNGUS IN CULTURE

Cultures of *Ceratostomella piceaperda* were developed by transferring slivers of blue-stained inner bark to malt agar. All the plantings produced yeast at first; later the blue-stain fungus appeared and grew over the yeast. The fungus was cultured with difficulty, the warm summer temperature at the laboratory (up to 30° C.) apparently being unfavorable for its growth. The cultures started from specimens collected at Grand Cascapedia in 1931 did not develop perithecia until the spring of 1932 and died during that summer. The cultures, started from a beetle collected in July 1933, began to develop perithecia 5 months later. A strain collected in 1930 has been in cultivation in the laboratory for 4 years. It is usually kept at 11° to 25°. The fungus developed mature perithecia in 14 days when growing on steam-sterilized pine (*Pinus caribaea*). It hydrolyzes boiled rice, producing a liquor. If it is combined with a yeast which also was isolated from *Dendroctonus piceaperda* infested bark, the hydrolyzation of the rice is stimulated, and a liquid is produced



FIGURE 8.—Bark of *Picea glauca* infested 15 days with *Dendroctonus piceaperda*. Blue stain caused by *Ceratostomella piceaperda* starting to spread from galleries.

that has a fruity aroma. The yeast alone barely grows on the rice. The presence of this yeast in a malt-agar culture markedly stimulates the growth and fruiting of the fungus.

A mature culture of *Ceratostomella piceaperda* grown on a malt agar slant is chaetura black. The aerial mycelium is usually hair brown at the top of the slant and black at the base. The surface of the culture has a velvety appearance due to the growth of conidiophores. These conidiophores become dark brown as they age and surround the perithecia, whose necks point up like shiny black thorns from the black substratum. Perithecia and conidiophores will be found developing ascospores and conidia at the same time, the fungus being unlike *C. pini* and *C. ips* in this respect.

#### MYCELIUM

The cultures used in the description of this fungus originated from a single conidium. The young mycelium is white, then cream-colored. The young hyphae, which measure  $2.6\mu$  to  $5.5\mu$  in diameter, form loose strands and change color quickly. A culture 4 days old, grown at  $25^{\circ}\text{C}$ ., is dark brown in the center. The hyphae walls thicken, and, as the culture ages, hyphae may be found having a diameter of  $17\mu$ .

#### CONIDIA AND CONIDIOPHORES

At  $25^{\circ}\text{C}$ . the first conidia appear on the hyphae 24 hours after the germination of a conidium. The conidia forming on the hyphae are hyaline, and obovoid or clavate (fig. 9, A). When germinating,

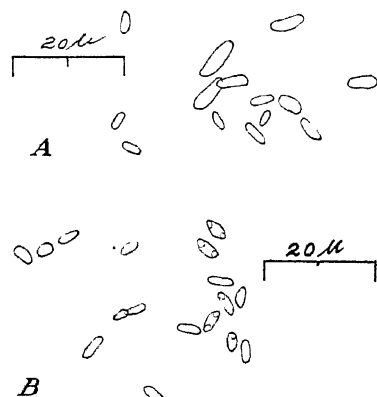


FIGURE 9.—*Ceratostomella piceaperda*: A, Conidia; B, ascospores.

the conidiophore ages, the color of the drop changes from white to yellow and then to brown. The conidiophores with their conidia seem to play an important part in the life cycle of this fungus, if vigor of growth is a criterion.

#### PERITHECIA

Beneath the bark of the spruce the perithecia develop in the insect galleries, their bases embedded in the bark tissues. In this situation the base is bare of hyphae. In culture the globular base develops

the conidium swells and often becomes globular. Some conidia bud like yeast. On others from 1 to 4 germination tubes have been seen. Simple short hyaline conidiophores appear that develop clavate conidia on their tips, but when 2 days old the fungus develops the characteristic conidiophores that resemble *Leptographium lundbergii* Lagerberg and Melin. The conidiophores are straight 5- to 7-septate brown hyphae, growing erect from the vegetative hyphae; near the tip a branch forms with hyaline subbranches, which develop the conidia at their tips. These conidia are held in a drop of a mucilaginous liquid on the top of the stalk (fig. 10). As

many-septate brown hyphae, which grow out into the air. These basal hyphae average about  $50\mu$  in length. The ostiole at the tip of the neck is without cilia (fig. 3, *E*). To the eye the perithecium is



FIGURE 10 —Conidiophores of *Ceratostomella piceaperda* from an 8-day-old culture.  $\times 175$ .

jet black. Some of the perithecia show a proliferation of the neck. The sizes of the perithecia are given in table 6.

#### ASCI

Only immature asci have been seen. They are hyaline and rather pear-shaped. Before the ascospores are ejected, however, the walls of the asci disintegrate. There are eight ascospores in an ascus.

#### ASCOSPORES

The ascospores are hyaline and ellipsoid (fig. 9, *B*). They have a thin coating of a mucilaginous substance which causes the spores to clump together on the tips of the perithecial necks, where they form a glistening mass.

TABLE 6.—Dimensions of perithecia of *Ceratostomella piceaperda*

Media	Fruits measured	Height of base			Width of base			Length of neck		
		Extreme range	Sextile range <sup>1</sup>	Mean	Extreme range	Sextile range <sup>1</sup>	Mean	Extreme range	Sextile range <sup>1</sup>	Mean
Malt agar.....	No. 76	90-350	140-240	197	80-340	130-250	193	130-860	270-650	μ 451
Rice agar.....	11	120-200	130-190	159	130-200	140-180	152	110-660	170-495	337
Wood.....	106	100-250	150-210	173	110-250	150-210	176	220-980	460-800	615
Total, range, or mean.....	193	90-350	150-230	181	80-340	140-220	181	110-980	360-660	535

<sup>1</sup> The sextile range discards the sixth of the measurements at each end of the extreme range, permitting an approximation of the frequency distribution.

#### TECHNICAL DESCRIPTION

#### *Ceratostomella piceaperda* n. sp.

Young conidia-producing colonies white to cream, changing to chaetura black; young hyphae hyaline, 2.6μ to 5.5μ in diameter, old hyphae brown, 6μ to 17μ in diameter; conidia hyaline, appearing first on hyphae, later on conidiophores, obovoid or clavate, extreme range 3μ to 11μ by 2μ to 4μ, mean 5.4μ by 3.2μ; conidiophores straight, brown, 5- to 7-septate, erect, branched, with conidia developing terminally on hyaline subbranches and collecting in a mucilaginous drop; conidiophores 170μ to 250μ high, stalk 4μ to 8μ in diameter; perithecia black, globose, hirsute; height of base, extreme range 90μ to 350μ, sextile range 150μ to 230μ, mean 181μ; width of base, extreme range 80μ to 340μ, sextile range 140μ to 220μ, mean 181μ; length of neck, extreme range 110μ to 980μ, sextile range 360μ to 660μ, mean 535μ; diameter of neck near base 45μ, at ostiole 25μ; ostiole without terminal cilia; asci ephemeral; ascospores 8, hyaline, ellipsoid; extreme range 3.6μ to 4.7μ by 1.0μ to 2.4μ, sextile range 3.9μ to 4.4μ by 1.9μ to 2.0μ, mean 4.3μ by 2.0μ.

On sapwood of *Picea glauca* infested with *Dendroctonus piceaperda* near St. Peters, Nova Scotia, Canada, and near Grand Cascapedia, Quebec, Canada.

#### DIAGNOSIS

#### *Ceratostomella piceaperda* sp. nov.

Colonis juvenilibus conidiferis albis cremeisve, tum brunneis-nigris cum peritheciis; hyphis juvenilibus hyalinis, 2.6μ-5.5μ, senescentibus brunneis, 6μ-17μ diam.; conidiophoris erectis, brunneis, ramosis, 170μ-250μ altis, 4μ-8μ latis; conidiis hyalinis, obovoideis vel clavatis, 3μ-11μ longis, 2μ-4μ latis, in hyphis primo apparentibus, apice ramulorum hyalinorum gestis, in guttām mucosam conglobatis; peritheciis nigris, globosis, hirsutis, 90μ-350μ altis, 80μ-340μ latis; rostellis 110μ-980μ longis, 25μ-45μ diam., ostiolo carente filamentis; ascis evanidis, octosporis; ascosporis hyalinis, ellipsoideis, 3.6μ-4.7μ longis, 1.0μ-2.4μ latis.

In ligno sapido *Piceae glaucae* *Dendroctono piceaperda* infestato, Nova Scotia et Quebec, Canada.

#### SUMMARY AND CONCLUSIONS

That certain bark-boring beetles carry specific fungi with them on their flights has been established by an accumulation of data collected during a number of years.

*Ceratostomella ips*, which causes the blue stain of sapwood of conifers on the Atlantic coast when infested with eastern species of *Ips* and which causes a blue stain of pine trees in western Japan, has been found to be associated also with the bark beetles *Ips emarginatus*, *I. integer*, and *I. oregoni*, which infest conifers on the Pacific coast.

Technical descriptions of two new fungi associated with bark beetles are given.

*Ceratostomella pseudotsugae* n. sp. is associated with *Dendroctonus pseudotsugae*, which infests Douglas fir and larch on the Pacific coast.

*Ceratostomella piceaperda* n. sp. is associated with *Dendroctonus piceaperda*, which infests spruce in eastern Canada.



# RELATION OF ACCESSORY GROWTH SUBSTANCES TO HEAVY METALS, INCLUDING MOLYBDENUM, IN THE NUTRITION OF *ASPERGILLUS NIGER*<sup>1</sup>

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## INTRODUCTION

The data presented in this paper were obtained initially in an attempt to develop a method for the complete removal, by extraction with alcohol, of heavy metals from the constituents employed in a nutrient solution for *Aspergillus niger* Van Tiegh. Though the method proved inapplicable for the purpose in mind, the results were extended and amplified once their significance in investigations on the accessory growth substances required by micro-organisms was realized. Correlation of the data on the heavy-metal requirements with those on the accessory growth bodies was considered also to be of importance.

The first observation on the existence of accessory bodies essential for the nutrition of the fungi was that of Wildiers (13),<sup>2</sup> who in 1901 announced the discovery of "bios", a hypothetical organic substance that he considered essential for the nutrition of yeast. This substance he found to be present in the filtrates of old yeast cultures and in apparently pure sucrose. It could be extracted from sucrose with 80-percent alcohol. The subsequent discovery of vitamins and of their importance in animal nutrition has led to the belief that "bios" functions similarly with the fungi. Investigators, however, have not agreed upon the necessity of "bios" and similar substances for the nutrition of fungi and bacteria, although the difficulties associated with the maintenance of pure cultures of micro-organisms in artificial media are not disputed. Effect of quantity of inoculum, dying out of cultures, and alterations in virulence and other functions are phenomena recognized by all.

## REVIEW OF LITERATURE

Of the voluminous literature on the accessory substances required by micro-organisms, mention may be made of a few recent papers. Hall, James, and Stuart (5) have reported that recrystallization of sucrose with alcohol eliminated a substance believed to be an organic nitrogen compound (bios) the presence of which is necessary for the growth of yeast. Extraction of sucrose with alcohol, on the other hand, results in the removal of another hypothetical organic substance ("coenzyme R"), not identical with bios, that is essential for the development of *Rhizobia*, according to Allison, Hoover, and Burk (2).

Claims of the existence of other accessory growth substances also have been made. Nielsen and Hartelius (6) state that *Rhizopus* *suinus* elaborates two such substances, one of which is identical with

<sup>1</sup> Received for publication Nov. 2, 1935; issued April 1936.

<sup>2</sup> Reference is made by number (italic) to Literature Cited, p. 448.

heteroauxin, regarded by Went (12) as a growth hormone of green plants. The second substance is "growth substance B", an accessory body for growth of *Aspergillus niger*. Vitamin B, the antineuritic growth factor, is considered by Schopfer (8) to be essential for the development of certain of the mucors. Peskett (7) has published an extensive review of the literature.

## METHODS

Both extraction with 95-percent alcohol and recrystallization with 80-percent alcohol have been employed for the removal of contaminants from sucrose in these investigations. The sucrose utilized was a highly refined sugar containing 0.002 to 0.0025 percent ash. Other chemicals were of reagent grade. The water was redistilled in Pyrex glassware. The organism was grown for 5 or 6 days at 34.7° to 35.1° C., in 200-cc Erlenmeyer flasks containing 50 cc of nutrient solution. The inoculum per flask averaged 0.05 mg (dry weight) of spore material.

Further details regarding the composition of the solutions used, the method of nutrient-solution purification, and other features will be found in former publications by the writer (10, 11). All solutions contained 5 percent of sucrose and the necessary salts.

The values tabulated are each the average of two duplicate cultures. The percentage yields are obtained by dividing the yield in the solution with an element omitted by the yield with the element present. That is, the yield in the complete nutrient, referred to as the maximum yield, is taken as equal to 100 percent in each experiment.

## GROWTH WITH SUCROSE PURIFIED BY MEANS OF ALCOHOL

The components of the nutrient solution were extracted with freshly distilled 95-percent alcohol for 6 hours or longer in a Pyrex extraction apparatus. The sucrose dissolved partially, the ammonium nitrate completely, while the dipotassium phosphate and the magnesium sulphate were practically insoluble. The ammonium nitrate was permitted to crystallize from the solvent, which was filtered off, and the crystals were dried at 103° C. to remove alcohol. The crystals of the other compounds were dried similarly. All were kept in a desiccator over anhydrous calcium chloride until required for use. The compounds retained the identical chemical formulas that they had originally except in the case of magnesium sulphate. Ignition of a sample of the latter compound after extraction with alcohol revealed that it had changed from heptahydrate to dihydrate; 0.38 g of the latter is formed from 0.6 g of the former.

Table 1 shows growth of the fungus in a nutrient solution (dibasic) composed of 1,000 cc of water redistilled in Pyrex glassware, 50 g of sucrose, 2 g of ammonium nitrate, 0.48 g of dipotassium phosphate and 0.6 g of heptahydrate (or 0.38 g of dihydrate) magnesium sulphate. One experiment is with unextracted ingredients, another with extracted sucrose but unextracted salts, and the third with components all of which had been extracted with alcohol. Iron, zinc, copper, and manganese, when added to the solution, were in amounts of 0.20, 0.14, 0.04, and 0.02 mg per liter, respectively.



TABLE 1.—*Effect of alcoholic extraction of the nutrient-solution<sup>1</sup> components upon the iron, zinc, copper, and manganese requirements of Aspergillus niger*

Heavy metal <sup>2</sup> omitted	Reagent compounds not extracted (initial solution, pH, 7.13)				Sucrose extracted (initial solution, pH, 7.05)				All components extracted (initial solution, pH, 6.99)			
	Yield	Proportion of maximum yield obtained <sup>3</sup>	Acidity at harvest	Sporulation <sup>4</sup>	Yield	Proportion of maximum yield obtained <sup>3</sup>	Acidity at harvest	Sporulation <sup>4</sup>	Yield	Proportion of maximum yield obtained <sup>3</sup>	Acidity at harvest	Sporulation <sup>4</sup>
	Mg	Percent	pH		Mg	Percent	pH		Mg	Percent	pH	
All.....	80.7	7.60	2.48	6, bl..	37.3	4.33	2.82	4, j----	29.6	3.59	3.08	2, bl
Fe.....	340.5	32.09	1.89	4, bl..	291.7	33.86	1.85	4, j----	422.7	51.73	1.79	6, bl.
Zn.....	111.5	10.51	2.24	6, bl..	60.4	7.01	2.61	4, j----	40.3	4.93	2.74	4, j.
Cu.....	959.9	90.45	2.44	2, bl..	780.4	90.60	1.82	2, w----	771.9	94.46	1.91	4, y.
Mn.....	821.0	77.37	1.94	2, bl..	672.8	78.11	1.64	2, w----	768.7	94.09	1.76	6, bl.
None....	1,061.2	100.00	2.48	6, bl..	861.4	100.00	1.75	4, j----	817.2	100.00	1.80	6, bl.

<sup>1</sup> The nutrient solution (dibasic) was composed of 1,000 cc of water redistilled in Pyrex glassware, 50 g of sucrose, 2 g of ammonium nitrate, 0.48 g of dipotassium phosphate, and 0.6 g of heptahydrate (or 0.33 g of dihydrate) magnesium sulphate.

<sup>2</sup> Heavy-metal concentrations were as follows: Fe, 0.20; Zn, 0.14; Cu, 0.04; and Mn, 0.02 mg per liter.

<sup>3</sup> Yield from complete medium with no metal omitted taken as 100 percent.

<sup>4</sup> Sporulation is indicated as 0 (sterile) to 10 (black with spores), and spore color by the initial letter or letters of the words jet, black, brown, tan, yellow, and white.

A distinct decrease in yield is shown in both cases as the result of extraction with alcohol. Not only is the yield diminished in the minus-heavy-metal and the minus-zinc cultures, but it is also lower in the full-nutrient cultures. Apparently the decrease in yield is due partially to the removal of zinc from the sucrose and the salts. Sufficient zinc is thus removed to cause a decrease in the percentage of maximum yield from 10.51 to 4.93 in the minus-zinc cultures. Additional trials with the full-nutrient solution revealed that the supply of zinc had become deficient and that the maximum yield averaged about 940 mg when the zinc removed from the sucrose (0.02 mg Zn per liter) was replaced, or approximately 13.2 percent less than with unextracted sucrose. The decrement in yield with the full-nutrient culture appeared to be dependent to some extent upon the duration of extraction, but was not due primarily to a deficiency of any of the known constituents of the nutrient solution.

Further studies were concerned, therefore, with the identification of the unknown material responsible for the diminution in maximum yield. The effects of 55 elements, including the 12 known chemical elements of the nutrient solution, were tested on the growth of *Aspergillus niger* in the nutrient solution under discussion, and in several other deficient nutrient solutions as well. The net result of these experiments here applicable may be briefly summarized by the statement that molybdenum is essential for growth and sporulation of this fungus. Its addition made up the deficiency in maximum yield resulting from the use of purified sucrose. A comparison of the results obtained with untreated sucrose, extracted sucrose, sucrose recrystallized once with 80-percent alcohol, and sucrose three times so recrystallized is given in table 2.

TABLE 2.—Effect of alcohol purification of sucrose upon the growth and development of *Aspergillus niger* in relation to role of heavy metals, with special reference to molybdenum

Heavy metal omitted <sup>1</sup>	Sucrose not purified (initial solution, pH 7.22)				Sucrose extracted 6 hours (initial solution, pH 7.12)				Sucrose extracted 6 hours (initial solution, pH 7.23) <sup>2</sup>			
	Yield	Proportion of maximum yield obtained <sup>3</sup>	Acidity at harvest	Sporulation <sup>4</sup>	Yield	Proportion of maximum yield obtained <sup>3</sup>	Acidity at harvest	Sporulation <sup>4</sup>	Yield	Proportion of maximum yield obtained <sup>3</sup>	Acidity at harvest	Sporulation <sup>4</sup>
	Mg	Percent	pH		Mg	Percent	pH		Mg	Percent	pH	
All.....	49.8	4.46	2.81	2, bl.	27.0	2.48	2.96	2, bl.	27.2	2.48	2.80	2, bl.
Fe.....	275.4	24.68	1.81	4, bl.	309.6	28.45	1.83	4, bl.	302.4	27.49	1.83	4, bl.
Zn.....	105.5	9.45	2.35	6, bl.	38.5	3.54	2.78	4, bl.	50.0	4.55	2.67	4, bl.
Cu.....	1,019.4	91.35	2.56	2, t.	1,059.3	93.35	2.78	2, t.	1,029.4	93.59	3.12	2, t.
Mn.....	934.4	83.74	1.81	4, bl.	930.6	85.51	1.88	4, bl.	814.3	74.03	1.80	2, bl.
Mo.....	1,018.1	91.15	1.88	4, bl.	1,004.3	92.28	1.90	6, bl.	984.3	89.19	1.93	6, bl.
None...	1,115.9	100.00	2.68	8, bl.	1,088.3	100.00	2.55	8, bl.	1,099.9	100.00	2.84	6, bl.

Heavy metal omitted <sup>1</sup>	Sucrose extracted 12 hours (initial solution, pH 7.17)				Sucrose recrystallized (initial solution, pH 7.06)				Sucrose recrystallized 3 times (initial solution, pH ?)			
	Yield	Proportion of maximum yield obtained <sup>3</sup>	Acidity at harvest	Sporulation <sup>4</sup>	Yield	Proportion of maximum yield obtained <sup>3</sup>	Acidity at harvest	Sporulation <sup>4</sup>	Yield	Proportion of maximum yield obtained <sup>3</sup>	Acidity at harvest	Sporulation <sup>4</sup>
	Mg	Percent	pH		Mg	Percent	pH		Mg	Percent	pH	
All.....	32.0	2.87	2.96	2, bl.	29.4	2.65	2.02	2, bl.	35.3	3.25	2.87	3, bl.
Fe.....	336.0	30.11	1.81	4, bl.	349.1	30.71	1.76	4, bl.	422.3	38.89	1.73	6, bl.
Zn.....	53.0	4.75	2.65	4, bl.	50.6	4.57	2.73	4, bl.	38.8	3.57	2.74	3, bl.
Cu.....	1,039.9	93.19	3.07	2, t.	997.6	90.07	2.94	2, y.	950.6	87.53	3.07	2, t.
Mn.....	844.7	75.70	1.82	2, bl.	956.7	86.38	1.82	2, bl.	998.5	91.94	1.85	2, bl.
Mo.....	881.1	78.96	1.86	4, bl.	962.2	86.87	1.84	6, bl.	1,000.4	92.12	1.94	7, bl.
None...	1,115.9	100.00	2.83	6, bl.	1,107.6	100.00	2.66	8, bl.	1,086.0	100.00	2.76	7, bl.

<sup>1</sup> The heavy-metal concentrations used were as follows: Fe, 0.20; Zn, 0.14; Cu, 0.04; Mn, 0.02; and Mo, 0.01 mg per liter, except that in experiments 3 and 4 the zinc content was 0.16 mg per liter.

<sup>2</sup> The extraction apparatus was thoroughly cleaned of residues from previous extraction.

<sup>3</sup> See footnote 3, table 1.

<sup>4</sup> See footnote 4, table 1.

The effects of purification of sucrose with alcohol at low impurity levels appear to be the same whether extraction or recrystallization is employed. They differ, however, with the individual heavy metal present in the sucrose. Zinc and molybdenum are removed partially by these procedures, although the results with the latter are rather erratic. Copper is removed to a slight extent, especially through repeated recrystallization. Iron and sometimes manganese, however, give definite indications of an increase in concentration in the sucrose through this procedure. It will be observed that the maximum yields are practically identical. It should be added, however, that filtration was omitted during the recrystallization process.

The results of the experiments with purified sucrose are more intelligible if it is kept in mind that the sucrose employed is of an exceptional grade of purity, containing not over 0.0025 percent ash. With sucrose of a significantly lower degree of purity the effects of purification become far greater. It should not be forgotten, moreover, that the comparisons usually made in studies on accessory bodies would correspond to the contrasts in the present investigation between

cultures containing sufficient nutrients for maximum growth and cultures deficient in more than one constituent.

Additional data bearing upon the need of *Aspergillus niger* for molybdenum are shown in table 3. The data are from solutions differing in composition and in treatment in order to avoid so far as practicable the possibility of error through the use of a single and perhaps abnormal solution. The cultures were grown for 5 days. The first two experiments were performed simultaneously, the addition of iron sulphate (25 mg per liter) in combination with calcium carbonate being practically without effect. The added iron is removed and possibly a trace of copper added. The results in these two experiments are quite uniform and serve to illustrate the experimental precision that may be attained with duplicate cultures. The results obtained in the next experiment demonstrate that the data obtained with Pyrex glassware are practically identical with those with quartz. Such differences as exist are probably due to the use of decantation instead of filtration in the experiment with quartz. In this experiment, water redistilled in quartz and quartz vessels were employed in all operations except measurement. In the last experiment equivalent amounts of magnesium nitrate and sodium sulphate were substituted for the magnesium sulphate of the dibasic nutrient solution. Comparison of the results with those in the first experiment of table 2 indicates the presence in the magnesium sulphate of significant amounts of iron, manganese, and particularly molybdenum.

TABLE 3.—*Effects of modifications in nutrient solution composition and treatment on the growth and development of Aspergillus niger with heavy metals, particularly with molybdenum*

Heavy metal omitted	Dibasic nutrient solution purified with $\text{CaCO}_3$ (pH 7.02)				Dibasic nutrient solution purified with $\text{CaCO}_3 + \text{FeSO}_4$ (pH 6.98)			
	Yield	Proportion of maximum yield obtained <sup>1</sup>	Acidity at harvest	Sporulation <sup>2</sup>	Yield	Proportion of maximum yield obtained <sup>1</sup>	Acidity at harvest	Sporulation <sup>2</sup>
	Mg 9.2	Percent 0.93	pH 3.33		Mg 10.8	Percent 1.06	pH 3.16	
All.....	20.7	2.10	3.10	1, 1.....	21.3	2.10	2.99	1, bl.....
Fe.....	16.7	1.70	3.14	1, 1.....	22.7	2.24	3.06	1, bl.....
Zn.....	744.3	75.58	1.87	2, t.....	807.7	79.55	1.93	2, t.....
Cu.....	771.4	78.33	1.80	2, bl.....	805.9	79.38	1.80	2, bl.....
Mn.....	901.2	91.51	1.71	4, 1.....	925.8	91.13	1.75	4, 1.....
Mo.....	984.8	100.00	2.13	4, bl.....	1,015.3	100.00	2.16	4, bl.....
None.....								

Heavy metal omitted	Dibasic nutrient solution purified with $\text{CaCO}_3$ in quartz vessels and culture flasks (pH 7.00)				Untreated dibasic solution <sup>1</sup> modified by substitution of $\text{Mg}(\text{NO}_3)_2$ and $\text{Na}_2\text{SO}_4$ for $\text{MgSO}_4$ (pH 7.22)			
	Yield	Proportion of maximum yield obtained <sup>1</sup>	Acidity at harvest	Sporulation <sup>2</sup>	Yield	Proportion of maximum yield obtained <sup>1</sup>	Acidity at harvest	Sporulation <sup>2</sup>
	Mg 7.4	Percent 0.78	pH 3.18			Percent 5.07	pH 2.53	
All.....	28.5	3.00	2.95	1, 1.....	52.4	10.86	2.00	4, bl.....
Fe.....	15.0	1.58	3.00	1, 1.....	174.4	7.77	2.04	6, bl.....
Zn.....	839.3	88.22	2.17	3, t.....	80.4	88.06	2.51	2, w.....
Cu.....	899.3	97.53	1.87	3, 1.....	916.9	72.96	1.81	2, 1.....
Mn.....	930.2	97.78	1.93	6, bl.....	754.5	65.55	1.72	2, bl.....
Mo.....	951.3	100.00	2.06	6, bl.....	877.9	65.55	2.36	8, bl.....
None.....					1,034.2	100.00		

<sup>1</sup> Equivalent amounts of  $\text{Mg}(\text{NO}_3)_2 \cdot 6\text{H}_2\text{O}$  and  $\text{Na}_2\text{SO}_4$  were substituted for  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ .

<sup>2</sup> See footnote 3, table 1.

<sup>3</sup> See footnote 4, table 1.

As a final check upon the necessity of molybdenum for the development of the fungus, three other salts of this element were tested. Besides the ammonium molybdate customarily employed, a sample of sodium molybdate from the same manufacturer was also used. In addition, samples of both these salts purchased from another manufacturer were tested in a similar manner. The results obtained with all molybdenum salts were quite similar and indicated the necessity for this element in the nutrition of *Aspergillus niger*.

#### GROWTH WITH MISCELLANEOUS ORGANIC SUBSTANCES

The results shown in table 4 were obtained in an attempt to determine the effects of traces of organic materials stated to contain accessory growth substances or of the alleged growth substances themselves upon the growth of *Aspergillus niger*. The absence of any increase in maximum yield (compare experiment 1, table 2) as a consequence of the addition of these materials would indicate that

TABLE 4—Effect of miscellaneous organic substances upon the growth of *Aspergillus niger* in a dibasic nutrient solution

Heavy metal <sup>1</sup> omitted	Heavy inoculum <sup>2</sup> used with solution (initial pH, 7.19)				Vitamin B <sup>3</sup> present in solution (initial pH, 7.18)				Vitamin C <sup>3</sup> present in solution (initial pH, 7.21)			
	Yield	Proportion of maximum yield obtained <sup>4</sup>	Acidity at harvest	Sporulation <sup>7</sup>	Yield	Proportion of maximum yield obtained <sup>4</sup>	Acidity at harvest	Sporulation <sup>7</sup>	Yield	Proportion of maximum yield obtained <sup>4</sup>	Acidity at harvest	Sporulation <sup>7</sup>
	Mg	Percent	pH		Mg	Percent	pH		Mg	Percent	pH	
All.....	45.9	4.30	2.75	6, bl.	34.2	3.43	2.88	4, bl.	30.0	2.91	2.81	4, bl.
Fe.....	419.1	39.27	1.78	6, bl.	154.7	15.52	2.08	4, bl.	242.3	23.47	1.88	4, bl.
Zn.....	87.5	8.20	2.41	6, bl.	36.2	3.63	2.81	4, bl.	51.7	5.01	2.68	4, bl.
Cu.....	855.3	80.14	3.56	4, t.	792.5	79.52	2.21	1, t.	910.3	88.19	2.54	1, t.
Mn.....	965.6	90.47	1.73	6, bl.	782.7	78.54	1.78	2, bl.	788.7	76.41	1.72	2, bl.
Mo.....	922.3	86.41	1.83	6, bl.	783.2	78.59	1.69	3, j.	792.2	76.75	1.68	4, j.
None....	1,067.3	100.00	3.12	8, bl.	996.6	100.00	2.48	6, bl.	1,032.2	100.00	2.54	6, bl.

Heavy metal <sup>1</sup> omitted	Yeast decoction <sup>4</sup> present in solution (initial pH, 7.15)				Malt extract <sup>5</sup> present in solution (initial pH, 7.17)			
	Yield	Proportion of maximum yield obtained <sup>6</sup>	Acidity at harvest	Sporulation <sup>7</sup>	Yield	Proportion of maximum yield obtained <sup>6</sup>	Acidity at harvest	Sporulation <sup>7</sup>
	Mg	Percent	pH		Mg	Percent	pH	
All.....	230.4	21.88	2.16	6, bl.	212.3	20.11	2.01	6, br
Fe.....	572.5	54.36	1.92	4, j.	409.8	44.41	1.84	4, j.
Zn.....	264.6	25.12	2.00	8, bl.	202.4	24.86	1.95	8, bl.
Cu.....	1,012.8	96.16	3.29	4, t.	984.1	93.22	3.28	4, v.
Mn.....	932.2	88.51	1.70	6, j.	952.7	90.24	1.77	6, j.
Mo.....	914.9	86.87	1.96	8, bl.	1,002.2	94.93	2.54	8, bl.
None....	1,053.2	100.00	2.96	9, bl.	1,055.7	100.00	2.96	8, bl.

<sup>1</sup> Heavy-metal concentrations were as follows: Fe, 0.20, Zn, 0.16, Cu, 0.04, Mn, 0.02, and Mo, 0.01 mg per liter.

<sup>2</sup> Inoculum 20 times standard or about 1.0 mg per flask.

<sup>3</sup> Vitamin B<sub>1</sub> or vitamin C at 5.0 mg per liter.

<sup>4</sup> Yeast decoction, 1.0 cc of a 1-percent solution per liter (filtered).

<sup>5</sup> Malt extract 10 cc of a 1.6-percent solution per liter (filtered).

<sup>6</sup> See footnote 3, table 1.

<sup>7</sup> See footnote 4, table 1.

growth of the "W" strain of *Aspergillus niger* used in these experiments is not dependent upon accessory growth substances. Owing to the writer's inability to obtain a culture of *Rhizopus suinus* at this time, no test of Nielsen's "growth substance B" could be made. It should be noted, however, that Nielsen's maximum yield for *Aspergillus niger* is  $1,168 \pm 68$  mg for 2.5 g of sucrose, while that obtained by the author with recrystallized sucrose and without accessory growth substances has been as high as 1,192.0 mg per 2.5 g of sucrose.

The data of table 4 indicate that growth increases obtained by investigators with accessory growth materials such as yeast extract, malt extract, and similar substances derived from organic materials are due, at least partially, to the presence of heavy metals in these substances. The experiments of table 4, in fact, may be considered as biological assays of the iron, zinc, copper, manganese, and molybdenum content of these materials. A crude approximation of the heavy-metal content introduced can be arrived at if proportionality in cause and effect is assumed. The difference in percentage maximum yields between the test solution and the control, divided by the similar difference between the maximum and the control, times the optimum concentration, equals the amount of heavy metal introduced.

#### AN OPTIMUM NUTRIENT SOLUTION

It was pointed out in a former publication (10) that the mineral constituents contained in the Pfeffer optimum solution and in the Raulin optimum solution were excessive in quantity because of the failure to include all heavy metals essential for the growth of *Aspergillus niger*. The optimum solution (dibasic), as determined by the writer, suffers from the same defect, since no molybdenum was added. Table 5 affords a comparison of these solutions and of a revised dibasic solution which includes molybdenum (0.02 mg per liter), as well as iron (0.20 per liter), zinc (0.18 per liter), copper (0.04 per liter), and manganese (0.02 per liter). The ammonium nitrate, dipotassium phosphate, and magnesium sulphate were used in amounts of 1.90 g, 0.35 g, and 0.25 g per liter, respectively. The yield at 35° C. is approximately 1,175 mg per 2.5 g of sucrose for a growth period of 5 days. The coefficient of utilization, therefore, is about 47 percent.

TABLE 5.—A comparison of optimum solutions giving maximum yields of *Aspergillus niger*

Solution	Total content of salts per liter	Heavy metals added	Milligrams per liter of indicated element				
			N	K	P	Mg	S
	<i>Grams</i>						
Pfeffer.....	17.50	Fe.....	3,500.0	1,435.0	1,140.0	247.5	325.0
Raulin.....	6.77	Fe, Zn.....	1,017.0	250.0	108.0	68.0	53.0
Dibasic.....	3.08	Fe, Zn, Cu, Mn.....	700.0	215.5	185.4	59.4	278.0
Revised dibasic <sup>3</sup> .....	2.50	Fe, Zn, Cu, Mn, Mo.....	695.0	157.1	161.3	24.8	232.5

<sup>1</sup> Phosphorus probably in excess.

<sup>2</sup> Sulphur in excess. Actual optimum about 20 mg per liter.

<sup>3</sup> Includes heavy metals in following concentrations: Mo, 0.02; Fe, 0.20; Zn, 0.18; Cu, 0.04; and Mn, 0.02 mg per liter; also  $\text{NH}_4\text{NO}_3$ , 1.90;  $\text{K}_2\text{HPO}_4$ , 0.35; and  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.25 g per liter.

## DISCUSSION

The evidence obtained with respect to the need of the fungus for molybdenum is quite convincing. The action of molybdenum is specific as judged by a comparison with that of salts of 54 other chemical elements, and apparently is not due to the presence of an impurity in the molybdenum compounds employed. Its presence aids both growth and sporulation and hastens development so that the maximum yield is attained a day earlier. The results, furthermore, cannot be attributed to any peculiarity of the nutrient solution, inasmuch as several other nutrient solutions gave similar results. Lastly, its inclusion in the formula of the optimum solution results in a decrease in the total quantity of salts per liter required for maximum growth from 3.08 g to 2.50 g, or about 19 percent.

The fact that growth is still appreciable even though no molybdenum has been added to the nutrient solution is without doubt due to unremoved traces of molybdenum. The spores used for inoculating the cultures also probably contain traces of this element. The same condition exists with respect to iron and zinc, and especially copper and manganese. In experiments reserved for publication in another connection it has been possible to obtain much greater differences in growth with and without addition of molybdenum to the nutrient solution. The yields are only about 10 to 12 percent of those in the complete nutrient solution, or poorer than those reported for iron, copper, and manganese in this paper.

Bortels (3, 4) has claimed that molybdenum and probably vanadium are necessary for the process of nitrogen fixation in *Azotobacter* and in *Rhizobia*. Schröder (9), moreover, considers molybdenum, as well as iron, zinc, copper, tungsten, silica, and perhaps calcium, essential for nitrogen fixation by *Azotobacter*. Though later investigators have noted also that growth is accelerated by molybdenum, the impression remains that a special connection exists between molybdenum and nitrogen fixation. It is doubtful, however, in view of the results with *Aspergillus niger*, whether the needs of these bacteria for molybdenum are limited to the process of nitrogen fixation. Molybdenum, in all probability, aids nitrogen fixation primarily because it is an element essential for growth and development, even though it may play a specific part in nitrogen metabolism.

The successive decreases in the quantities of nitrogen, potassium, phosphorus, magnesium, and sulphur required for maximum yields, with more complete provision of the essential heavy metals,<sup>3</sup> probably constitute a factor in agricultural practice as well as in nutrient-solution studies. On the other hand, though the chemical elements required only in minute quantities may be assumed to be present in the majority of soils in amounts sufficient for maximum yield of green plants, it seems likely that at least some if not many exceptions occur. The determination of the chemical element essential in minute quantities for plants cannot be considered, therefore, as a problem of only abstract importance.

No evidence could be obtained that *Aspergillus niger* ("W" strain) requires the presence of accessory growth substances for its normal growth and development. The diminution in yields of this organism, obtained as a result of alcoholic purification of the sucrose of the

<sup>3</sup> The presence of molybdenum, as well as of iron, zinc, copper, and manganese, has been detected repeatedly in the ash of tobacco plants by the effects of these metals on the growth of *Aspergillus niger*.

nutrient solution, proved to be due to the removal of zinc and of molybdenum. That the procedures adopted are comparable to those recommended by others for work of this character would seem certain, since as the result of their employment the nutrient solution proved entirely incapable of supporting the growth of various *Rhizobia* and *Saccharomycetes* except upon the addition of other substances.

On the other hand, the use of organic materials or of substances derived from such materials may lead to exaggerated responses because of the concomitant addition of heavy metals with the supposed accessory growth factors. The presence in such materials of heavy metals essential for growth is definitely indicated in these experiments and should be taken into account. The employment, for example, of a culture medium considered by Allison and Hoover (1) to be suitable for demonstrating the necessity of "coenzyme R" for *Rhizobia* involves the use of an excess of calcium salt at pH 6.9 from which the clear solution is siphoned. This procedure happens to be a variant of the writer's method of nutrient-solution purification for the removal of heavy metals. Utilization of this treatment suggests the partial influence of heavy metals on the increases in growth attributed to "coenzyme R."

The absence of evidence in confirmation of the results of Nielsen and Hartelius (6) with *Aspergillus niger* and "growth substance B" requires further elucidation. It may be, of course, that the strain employed by Nielsen and Hartelius would respond exactly as does the "W" strain under these conditions. This might imply that the hypothetical "growth substance B" is simply a mixture of essential heavy metals, perhaps in organic combination. On the other hand, there is definite evidence in the literature that the individual accessory growth substances are required in markedly different degree even by forms in closely related genera. That this would also apply to species of the same genus and even to strains of the same species in some cases is not improbable. Another interpretation is possible, however. Since the necessity for these specialized bodies would imply a loss in physiological functions, namely, the ability to synthesize various organic chemical nuclei essential for metabolism, its effect would be to induce specialization within a more or less restricted habitat. That is to say, an organism like *Aspergillus niger*, which seems to grow quite freely on all types of substrata, would not be apt to require accessory growth substances unless these are universally present in practically all types of organic materials.

#### SUMMARY

The growth and development of *Aspergillus niger* in synthetic nutrient solutions have been studied with special reference to the influence of heavy metals and accessory growth substances. The results indicate that accessory growth substances are not required for the nutrition of *A. niger* ("W" strain). The decreases in yield of this fungus consequent upon utilization of sucrose purified by alcohol (the procedure for removal of "bios" and "coenzyme R") are due almost entirely to the removal of zinc and of molybdenum. Extracts of organic materials, also, such as yeast decoction or malt extract, when used as a source of assumed accessory growth substances, may cause an increase in growth because of the essential heavy metals they contain.

Molybdenum is essential for the growth and development of *Aspergillus niger*, and therefore cannot be associated exclusively with the process of nitrogen fixation in bacteria. An optimum solution (5 percent sucrose) for the growth and development of this fungus, containing iron, zinc, copper, manganese, and molybdenum, as well as the usual constituents, has a total salt content of 2.50 g per liter. Experimental precision within 1 percent in the values of percentage maximum yield is attainable with duplicate cultures in replicate trials.

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# CHRYSID SHANGHAIENSIS SMITH, A PARASITE OF THE ORIENTAL MOTH<sup>1</sup>

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## INTRODUCTION

*Chrysis* (*Pentachrysis*) *shanghaiensis* Smith is a parasite of the oriental moth, *Cnidocampa flavescens* (Walk.) (3),<sup>3</sup> in the Orient, its native habitat, but attempts to establish it in the area around Boston, Mass., infested with this moth have been unsuccessful. These attempts were made by Fernald (5) in 1917 and 1918, when he liberated adults that issued from cocoons of the oriental moth parasitized by *C. shanghaiensis* received from China. Although 6 percent of the cocoons collected in 1919 were parasitized, no recoveries from these liberations have been made since that time.

In recent years several shipments of oriental moth cocoons have been made to this country from Japan with the object of introducing parasites that might aid in the control of this pest. Among those received in 1932 was *Chrysis shanghaiensis*.<sup>4</sup> This paper presents the results of two seasons' work with the species and notes on its habits. It was intended to conduct experiments the following year, but the low temperatures in the winter of 1933-34 killed all the hibernating larvae of *Chrysis*.

## GENERAL HABITS OF CHRYSIDIDAE

The majority of the chrysidids are parasites orinquilines of solitary wasps and bees. The females lay eggs in the nests being provisioned by the rightful owner, and the larvae developing from these eggs eat the provisions, or the larvae of the wasps and bees that develop on the provisions, or both.

Maréchal (12), describing the development of *Chrysis ignita* L. on *Odynerus parietum* L., stated that the larva of *Chrysis* consumes the partly developed larva of *Odynerus* and the remaining provisions in the nest.

Ferton (6, ch. 5) observed that some species of *Chrysis* prevent competition from their own kinds by the destruction of all eggs by the first larva to hatch.

Hicks (8) described the habits of *Chrysis pacifica* Say, parasitic on the mature larvae of *Aleidamea brachyodonta* Ckll. The host cell is stored with pollen. The larvae of *Chrysis* were found on host larvae

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<sup>3</sup> Reference is made by number (italic) to Literature Cited, p. 457.

<sup>4</sup> These cocoons were sent to the Bureau of Entomology laboratory at Melrose Highlands, Mass., by L. B. Parker, of the Japanese and Asiatic beetle investigations, who had collected them on the island of Shikoku, Japan.

of various sizes, but little or no feeding occurred until the host reached the prepupal stage.

Janvier (9, p. 287), working with *Tetrachrysis carinata* Guer., an external parasite of the mature larva of *Odynerus humeralis* Hal. in Chile, found that feeding host larvae are not attacked but only those that have spun their cocoons. The male *Tetrachrysis*, after feeding for several days on flowers, searches out a host cell and makes a hole in it. It is then revealed that this cell contains a female *Tetrachrysis*. The female is seized by the thorax and pulled out and mating is then accomplished.

Chapman (2) recorded observations on *Chrysis bidentata* L. (*viridula* L.), a solitary, external parasite of the larva of *Odynerus spinipes* (L.) Spin. This species attacks mature larvae of the host in cells that are incompletely protected in unfinished burrows. From 6 to 10 eggs are placed on a host larva, but there is no evidence that more than 1 egg ever hatches.

Bordage (1), observing the development of *Chrysis lusca* var. *concinna* Grib., a parasite in the cells of *Sceliphron*, noted that the egg of *Chrysis* hatches after the host egg hatches and that a combat occurs between the primary stages of the parasite and the host. If the host egg does not hatch, the parasite larva dies without touching the mass of food available.

Maneval (11) cited the only instance of internal parasitism recorded for Chrysididae. *Chrysis neglecta* Shuck. is a solitary, internal parasite of the larva of *Osmia villosa* Schenck. The first-instar larva of *Chrysis* enters the host larva and develops as an internal parasite.

*Chrysis shanghaiensis* is an exceptional chrysidid, as it is a parasite of a lepidopterous host. The writer has seen no reference to any other chrysidid acting as a parasite of a lepidopteron.

#### LITERATURE ON CHRYSIS SHANGHAIENSIS

*Chrysis shanghaiensis* was described in 1874 by Smith (15). Moc-sáry (13, p. 522) listed *shanghaiensis* as the only chrysidid parasitic on a lepidopterous host. Joannis (10) noted the presence of *C. shanghaiensis* in cocoons of the oriental moth received from China from P. R. Gaudissart, a missionary. The adults were determined by Gribodo, who published notes (7) on some of the habits of the species which he observed in material received from Joannis. Du Buysson (4) presented the distribution of the species, described the poison glands of the female, and synonymized *C. himalaensis* Mocs. and *C. crassicutra* Mocs. with *C. shanghaiensis*. The early attempts of Fernald (5) to establish *C. shanghaiensis* in the area around Boston infested with the oriental moth have already been mentioned. Piel and Covillard (14), in a paper on the oriental moth and its parasites, devoted considerable space to a discussion of *C. shanghaiensis*, making many valuable observations on the habits of the species and describing some of the larval and pupal development.

#### DISTRIBUTION

According to Piel and Covillard (14), *Chrysis shanghaiensis* is a southern form, and they recorded it from India, the Himalayas, China, Siam, Sumatra, Java, and Gorontalo. It is also found in

Japan. *Cnidocampa flavescens* is recorded from the Orient, eastern Siberia, China, Chosen, and Japan. Since the range of *C. shanghaiensis* does not coincide with that of *C. flavescens*, it is probable that this parasite can reproduce on other Eucleidae found in these regions.

## HABITS

### BEHAVIOR OF ADULTS

Adults of *Chrysis shanghaiensis* respond quickly to changes of light and temperature. During cool weather or in subdued light they congregate in places that afford protection and concealment. In the laboratory they are found between the cloth of the cage and its wooden framework, beneath papers upon which the food is placed, or in empty host cocoons. In bright light and at higher temperatures their activity increases. The antennae vibrate continuously, and at intervals the wings produce a rather high pitched buzzing sound. This is accomplished without spreading the wings and is apparently due to a rapid friction between them. The adults are capable of strong and rapid flight.

Like the adults of various other Chrysididae, they defend themselves against attack by wasps or bees by curling the abdomen under the thorax so that no soft parts of the body are presented to the sting of the aggressor.

### MATING

Adults of *Chrysis shanghaiensis* mate readily in cloth cages, or in wooden boxes with glass tops, on clear, warm days, and also, although more slowly, when there is no sunshine. The males are very active and pursue the females vigorously, vibrating their wings so rapidly that they appear as a blur. This causes a humming sound that can be heard several feet away. In 15 observed matings the act lasted from 6 to 14 minutes, the average being 8 minutes. None of the females mated more than once, but two males mated twice. Attempts to obtain further mating by these males were unsuccessful.

### METHOD OF ATTACK

The cocoons that were attacked in the laboratory had been collected in the field by cutting the twigs upon which they were spun, or had been formed in the laboratory by last-instar larvae collected in the field. Cocoons on twigs of the diameter of a pencil or larger were attacked most readily.

The female *Chrysis* approaches a cocoon and examines it with her antennae. If it proves satisfactory, she takes a position on the twig facing either end of the cocoon. If the cocoon has been spun in a crotch formed by two twigs, she can attack the cocoon from the side, but normally she prefers the ends. She grasps the twig firmly, the posterior legs slightly arched and the anterior legs placed somewhat forward, and applies the mandibles about one spot on the cocoon. While chewing a hole in the cocoon, she braces the five pointed projections on the tip of her abdomen against the surface of the twig. She holds her body rather rigidly, but as the mandibles are scraping about the point of attack she turns her head first one way and then the other, making striations that completely surround the central point. When a hole is broken through the cocoon, she places the point of one

mandible in the hole and that of the other firmly on the surface of the cocoon away from the hole. The action then is a rasping and pulling one, working from the center toward the mandible which is used as an anchor.

In seven instances this operation required from 10 to 30 minutes. In the majority of cases observed, however, it consumed nearly 30 minutes, the 10-minute period being exceptional.

The wall of the host cocoon is strengthened by a gradual hardening of the cementing substance used in its formation. It was noted that cocoons used a few hours after the host larva had completed the spinning were not satisfactory for *Chrysis*. Instead of chewing a small, round hole, the parasite would tear a jagged hole in the cocoon and leave without ovipositing. This did not occur when cocoons were held for 24 hours or more before they were placed with the *Chrysis*.

#### OVIPOSITION

In ovipositing, the female reverses her position on the twig and faces away from the cocoon. The long ovipositor is exerted, and she attempts to locate the hole that she has made in the cocoon. In a few of the cases observed the hole was apparently not suitable, for the female turned and enlarged it. When the ovipositor is fully inserted, the tip of the abdomen is practically resting on the cocoon.

The ovipositor of *Chrysis* is about 7 mm long and is very flexible. Observations made through an opening in the side of a cocoon showed that the female extends the ovipositor around the larva on all sides before depositing an egg. Apparently poison is injected during this prodding, for the host larva becomes paralyzed. The act of egg deposition requires from 6 to 30 minutes, the average duration being 15 minutes.

After depositing the egg, the female again applies her mandibles to the cocoon and scrapes the surface toward the hole. The loosened material she mixes with an oral secretion and packs into the hole, sealing it. The area surrounding the sealed hole is marked with fine radiating striations to a distance of about 1 mm. The sealing of the hole requires from 9 to 37 minutes, the average being 20 minutes. Rarely a female will leave the cocoon without sealing the opening.

#### EGG CAPACITY

Seven females deposited 79 eggs from August 26 to November 7 under laboratory conditions, or an average of 11.3 eggs per female. This seems to be a small number for such a numerous species, but it is slightly larger than the 8- to 10-egg average determined by Piel and Covillard (14) for the group of females that they studied. It is possible that this species deposits more eggs under natural conditions.

#### DEVELOPMENT

##### EGG

The egg of *Chrysis shanghaiensis* (fig. 1) is deposited externally on the body of the host larva within its cocoon and adheres only slightly unless it is deposited on the venter, which in itself has adhesive qualities.

A freshly deposited egg is dull white, elongated, rounded, and slightly curved, bearing a very short petiole at the cephalic end. The surface is finely roughened by crystal-shaped protuberances, which are more clearly seen on an egg from which the larva has hatched.

Measurements of 10 eggs showed them to range from 2.1 to 2.5 mm in length and from 0.4 to 0.5 mm in width. Hatching occurs about 4 days after deposition.

#### FIRST-INSTAR LARVA

The newly hatched first-instar larva (fig. 2) measures 1.5 to 1.9 mm in length and 0.5 to 0.6 mm in width. It consists of a large, squarish head and 13 body segments which, viewed laterally as in figure 2, vary slightly in width for two-thirds the length of the body, then taper off to a bluntly bifurcate posterior segment which is used as an aid to locomotion. The body is white at first, but it soon becomes creamy yellow from the ingested food. The pleura bear fleshy lobes.



FIGURE 1.—Outline drawing of an egg of *Chrysis shanghaiensis*.  $\times 20$ .

The endoskeleton of the head, upon which the mandibles articulate, is well sclerotized. The labrum, labium, and maxillae are fleshy and prominent. The mandibles are shown in figure 3, A.

The tracheal system consists of two lateral trunks starting in the prothoracic segment and extending posteriorly, where they are joined in the twelfth body segment. The spiracles are present in nine seg-

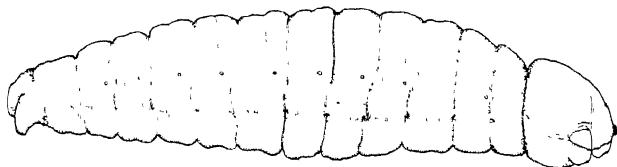


FIGURE 2.—Partially grown first-instar larva of *Chrysis shanghaiensis*.  $\times 53$ .

ments, the mesothoracic and the first eight abdominal segments. In the metathoracic segment there are well-developed spiracular branches but no apparent spiracular openings.

The first stadium averages 4 days in length.

#### INTERMEDIATE INSTARS

There are three intermediate instars. The exuviae can be found just behind the feeding larva or still attached to it. The changes in the body in these instars are slight. It becomes relatively stouter, the fleshy lobes on the pleura become more pronounced, and the last segment loses its bifurcate character with the first molt. The head of the larva becomes more hemispherical with each molt and the endoskeleton more heavily sclerotized. The mandibles show considerable change, as illustrated in figure 3, B, C, and D.

In the second instar the metathoracic spiracular opening appears, and in the remaining instars there are 10 pairs of open spiracles.

Each of the intermediate stadia averages 3 days in length.

## LAST-INSTAR LARVA

When the last-instar larva has completed its growth, there is little left of the host except the sclerotic parts of the head and some body spines. The larva spins a golden silken cocoon, excluding the remains of the host and the meconium. Figure 4 shows an average full-

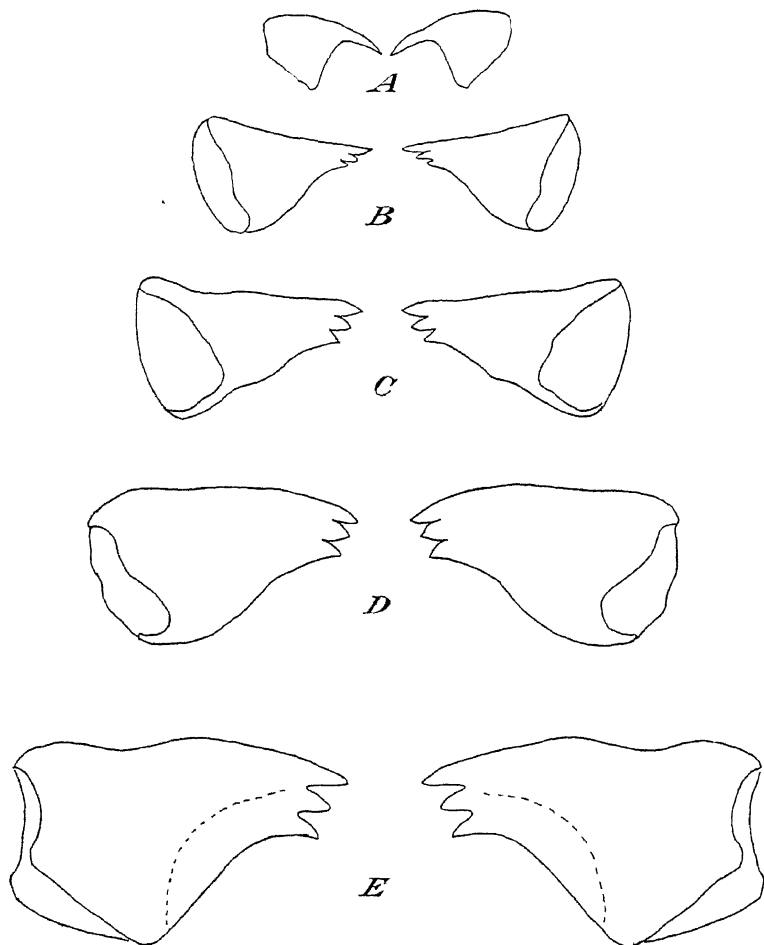


FIGURE 3—Outline drawings of larval mandibles of *Chrysos shanghaiensis*: A, First instar; B, C, and D, intermediate instars; E, last instar.  $\times 112$ .

grown larva, which measured 10.5 mm in length and 5.2 mm in width. The last-instar larva is creamy yellow in color.

Figure 5, showing the head of a mature larva, is given in some detail, for it is representative of the head characters found in the other instars. The lower part, including the labium and the maxillae, is sharply set off from the upper part, bearing the labrum, the mandibles, and the antennae. The sclerotized features of the labial and maxillary regions of the head are hidden by the fleshy labium and maxillae. The large brownish mandibles are shown in figure 3, E.

## PUPA

Pupation occurs the latter part of June. The pupa is white at first. The eyes and the ocelli soon darken, and then the body shows a faint greenish reflection which gradually becomes more pronounced

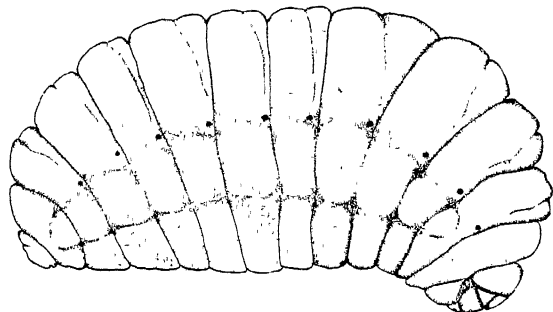


FIGURE 4.—Mature last-instar larva of *Chrysis shanghaiensis*.  $\times 7$ .

and finally becomes a deep bluish green with iridescent reflections. This gradual coloration progresses from the pronotum and the dorsum of the mesonotum to the metathorax, the abdomen, and finally to the legs. The pupal stage lasts a month or more, the first pupa having been noted on June 26 and the first adult on July 26.

## LONGEVITY

The adults of *Chrysis shanghaiensis* live very well in confinement on food consisting of either honey and water (1 to 4) or lump sugar and water, the water being supplied on sponges.

Piel and Covillard (14) found that the males lived 12 to 15 days and the females more than a month under conditions in China.

In experiments at this laboratory adults lived much longer. In 1932 the issuance of adults started on July 20 and extended to August 4, and host cocoons were attacked from August 11 to September 22. In 1933 the issuance of adults started July 26 and extended to August 24. Aside from two males and one female that died a short time previously, six dead males were removed on October 10, 47 days after the last adult issued. No more dead were removed until October 17, when two females died. The last adult died January 20, 1934. The length of life would be considerably shortened in nature because of the cold weather. This period of survival was possible under laboratory conditions because the adults were placed in a heated insectary. This record includes 97 adults.

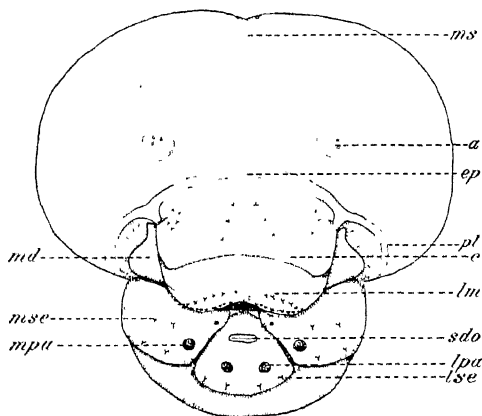


FIGURE 5.—Head of mature larva of *Chrysis shanghaiensis*,  $\times 36$ . a, Antenna; c, clypeus; ep, epistoma; lpa, labial palpus; lse, labial seta; lm, labrum; md, mandible; mpa, maxillary palpus; mse, maxillary seta; pl, pleurostoma; sdo, suture; se, orifice of silk duct.

## SEASONAL HISTORY

Piel and Covillard (14) found two generations of both host and parasite in China, the first generation of *Chrysis* appearing in the latter part of June and the second generation in August. In this laboratory the parasites overwintered as mature larvae within the host cocoons, the adults appearing late in July and early in August. The adults mated soon after issuing and attacked the host larvae within cocoons. The larvae became mature about 20 days after egg deposition, and each spun a golden, silken cocoon within the cocoon of its host and remained in this condition throughout the winter.

*Chrysis* was found in all stages of larval development until cold weather stopped the activity of the adults.

## INTERRELATIONS OF CHRYSIS AND CHAETEXORISTA

Studies were begun on the interrelations of *Chrysis shanghaiensis* and *Chaetexorista javana* B. and B., a tachinid parasite of the oriental moth that was established in the infested area in 1929 and 1930, but the extreme cold weather during the winter of 1933-34 killed all the material that was held in hibernation, making it impossible to continue this work.

These incomplete experiments indicated, however, that competition exists between these species. Both species develop on the host larva within its cocoon, *Chaetexorista* internally and *Chrysis* externally. A larva of *Chrysis* feeding externally on a host larva containing a second-instar larva of *Chaetexorista* usually matured at the expense of *Chaetexorista*. In some instances *Chaetexorista* developed into the third instar, which is normally attained after overwintering. This may have been due to the stimulation resulting from the feeding of the larva of *Chrysis*. In such cases the larva of *Chaetexorista* usually matured and formed a puparium, sometimes imperfect or larviform, and *Chrysis* died from lack of food. Some host cocoons were found to contain dead larvae of both species.

## FACTORS LIMITING THE EFFECTIVENESS OF CHRYSIS

Piel and Covillard (14) discussed a chalcid parasite, *Eurytoma monemae* Ruschka, which depends upon *Chrysis* for its ability to oviposit within a cocoon of the oriental moth. This parasite oviposits through the plug of material with which *Chrysis* seals the opening in the cocoon made during oviposition. These investigators infer that *Chrysis* is overcome by *Eurytoma* in such instances.

At this laboratory several larvae of *Chrysis* in cocoons that the female had failed to seal after oviposition were devoured by small chalcid larvae which proved to be *Mellitobia* sp. It is believed that *Mellitobia* would be unable to oviposit within a normal cocoon.

In December 1933 and early in 1934 the temperature in the laboratory yard was as low as  $-18^{\circ}$  F. This temperature killed all the larvae of *Chrysis* hibernating within host cocoons held in an unheated insectary. During the two previous seasons the lowest temperature was  $-4^{\circ}$  F., which allowed successful hibernation.



## SUMMARY

*Chrysis* (*Pentachrysis*) *shanghaiensis* Smith is a parasite of the oriental moth, *Cnidocampa flavescens* (Walk.), a pest of various shade and fruit trees. The oriental moth is established in and around Boston, Mass. Parasitized material from Japan yielded this chrysidid parasite, and studies concerning its biology were conducted. *C. shanghaiensis* is the only known chrysidid that is a parasite of a lepidopterous host.

The adults of *Chrysis shanghaiensis* are very active and capable of strong flight. The females mate readily and attack the larvae of the oriental moth by chewing a hole in the cocoon, depositing an egg in it external to the host larva, and then sealing the hole. The egg hatches in about 4 days, the larvae complete the first stadium in an average of 4 days, and the second, third, and fourth stadia in an average of 3 days each. The last-instar larva encloses itself in a golden silken cocoon, where it remains throughout the winter. The larva pupates during the latter part of June, and the adults emerge late in July and in the first part of August.

The adults live very well in captivity. Of 97 adults that emerged between July 26 and August 24, only 9 had died 47 days after the last adult emerged. The last adult died on January 20, nearly 5 months from the termination of issuance.

Uncompleted experiments on the interrelations of *Chrysis shanghaiensis* and *Chaetoxorista javana* indicated that some competition existed when both species attacked the same host larva.

One case of parasitism at the laboratory was traced to *Mellitobia* sp., but this occurred only because the oviposition hole in the cocoon was not sealed. The low temperatures of the winter of 1933-34 proved too severe for the *Chrysis* larvae held at the laboratory, and no doubt such temperatures would limit the survival of this insect in the vicinity of Boston.

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# THE INFLUENCE OF VARYING AMOUNTS OF WATER-SOLUBLE PHOSPHORUS IN DIFFERENT SOIL TYPES ON THE RESPONSE OF CULTIVATED CROPS<sup>1</sup>

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## INTRODUCTION

A low content of available nutrient elements in the soil is reflected in the growth of the vegetation, and often in the welfare of animals grazing over such areas. Phosphorus appears to be one of the elements deficient in the forage growing on some of the fine sandy loam soils in Florida. Lack of phosphorus results in the condition known as "sweeny" (phosphorus deficiency) in cattle. This condition does not occur on the coarser textured soils of the same locality.

The object of this investigation was to determine the response of different plants to varying amounts of phosphorus in soils of varying texture. Virgin samples of Orangeburg fine sandy loam were used as typical of the phosphorus-deficient soils, while Norfolk sand and Norfolk fine sand were used to represent types on which sweeny does not occur.

## REVIEW OF LITERATURE

The content of water-soluble phosphorus in sands was shown by Bryan (3)<sup>2</sup> to be much higher than that for sandy loams and loams even under conditions of heavy fertilization. This was due to the greater amounts of phosphate-fixing agents, iron and aluminum, in the finer textured soils and was intensified by the removal of soil bases through leaching processes. Because of this high fixing power of most humid soils the range of soluble phosphorus in the soil is limited. This has been shown by a number of investigators (5, 7, 8), especially with fine-textured soils, even regardless of frequent additions of phosphates.

Becker, Neal, Shealy, and York (2) found that the phosphorus content of the native forage plants growing on the fine sandy loams was less than that of the plants growing on coarser textured soils in the same locality. The level of phosphorus in the first instance was less than that required for the welfare of cattle. Theiler, Green, and Du Toit (11), Eckles, Becker, and Palmer (4), Scott (9), and others have found that a deficiency of phosphorus in cattle was due to a deficiency of this element in the forage vegetation. Mather (6) showed that phosphate fertilization of sands and peats increased the phosphorus content of the vegetative portion of forage, most of the increase being in the inorganic fraction. The results of Weidemann (13) confirm the increase of phosphorus in the vegetative portion when heavy fertilization is practiced.

<sup>1</sup> Received for publication Oct. 1, 1935; issued April 1936. The work reported in this article is one phase of the investigation of phosphorus deficiency in cattle as it occurs in Florida, and not only verifies the deficiency of phosphorus in the forage grown on affected areas, but indicates that excessive phosphate fertilization would be required to produce "protective" forage.

<sup>2</sup> Reference is made by number (italic) to Literature Cited, p. 466.

In the veld soils of South Africa, rainfall is often the limiting factor (11) so that phosphate additions on the soils where cattle suffer from a deficiency of the element do not give consistent increases in yields of forage. Stokes, Warner, and Camp (10) found phosphate fertilization to give little or no increase in yield of corn on the light, sandy soils of central Florida, while marked increases were secured on the heavier soils of northwest Florida. These latter soils are in the area where cattle suffer from phosphorus deficiency.

In general, fine-textured soils have a low content of water-soluble phosphorus. This may be increased by phosphate fertilization, but to a lesser extent than in the coarser textured soils. Phosphate fertilization may increase the phosphorus content of forage, and thus prevent the deficiency in cattle. But whether or not this would be economical in range soils remains to be shown.

#### METHOD OF PROCEDURE

The soils were placed in glazed, 2-kg earthenware jars and treated with superphosphate at rates varying from 0 to 8,000 pounds per acre. The cultures also were given a uniform treatment of 500 pounds of gypsum and 200 pounds of dolomitic limestone per acre. These materials were mixed thoroughly with the soils, which were allowed to stand for 12 weeks with weekly additions of water, in order to permit the soil and the amendments to reach equilibrium. At the end of the 12 weeks, nitrogen was added at the rate of 100 pounds each of nitrate of soda and calcium nitrate, and potash as sulphate at the rate of 75 pounds per acre. Mustard (*Brassica*), vetch (*Vicia*), and sorghum (*Holcus*) were used to measure the availability of the phosphorus in the soils. All series were replicated.

Mustard was planted first and water added as needed. During the growth of the crop, soil samples were taken at three intervals, and the water-soluble phosphorus was determined colorimetrically in 1 to 5 soil-water extracts (12). At the end of 60 days, the plants on each culture were cut, dried, and weighed.

Following the removal of the mustard crop, the soils in each jar were mixed, the fibrous roots removed, additions of nitrogen and potash made, and the cultures planted to vetch. The crop was allowed to grow, as in the case of the previous crop of mustard. The water-soluble phosphorus was also determined as before. At the end of 4 months the vetch was cut, dried, and weighed, and the phosphorus content determined by standard methods (1).

Following the crop of vetch, the soils were again mixed; potash and nitrogen were added as before; and sorghum was planted. However, the Norfolk sand was omitted. This crop was grown for 60 days, before the photographing and the taking of growth records. The phosphorus content of sorghum was determined as in the case of vetch.

For comparison a second series of soils was planted to sorghum, Norfolk fine sand and Orangeburg fine sandy loam being used. As with the other cultures, superphosphates were added in varying amounts, but in this case the seed was planted immediately following the application of the phosphate. Nitrogen and potash were added and the plants allowed to grow for 40 days.

## RESULTS

The oven-dry weights of mustard and vetch as affected by the water-soluble phosphorus in the soils are given in table 1. All values given are the average of two or more replications. The relative growth of all the crops with different amounts of phosphorus is shown in figures 1, 2, and 3. Examination of the data shows that the response of mustard and vetch to phosphates on Norfolk sand was small as compared with the response on the Orangeburg fine sandy loam. The response to phosphorus on the Norfolk fine sand was intermediate between that obtained on the other soils. The growth of mustard increased with increase in phosphate applications up to 1,000 pounds

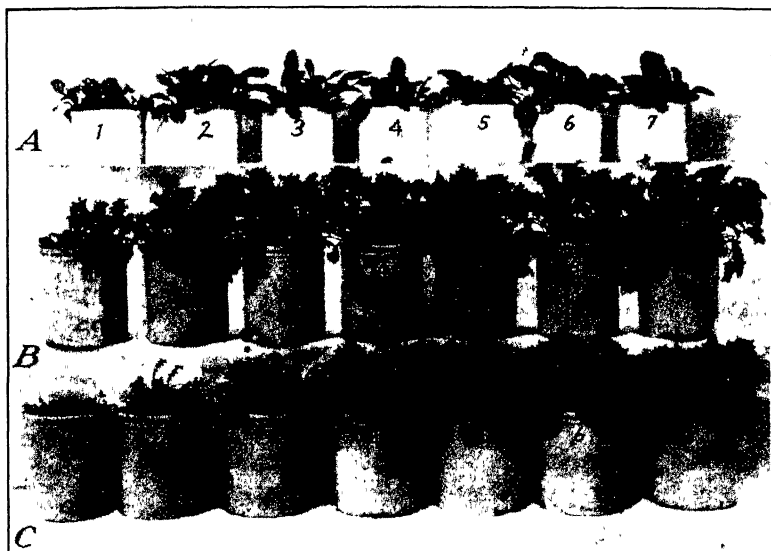


FIGURE 1—The effect of different amounts of phosphorus upon the growth of mustard in. *A*, Norfolk sand; *B*, Norfolk fine sand; *C*, Orangeburg fine sandy loam. All cultures treated alike except for phosphorus additions. Nos. 1 to 7 received 0, 500, 1,000, 1,500, 2,000, 4,000, and 8,000 pounds of superphosphate per acre, in numerical order.

per acre on Norfolk fine sand, while the growth increased on Orangeburg fine sandy loam up to 4,000 pounds per acre. It appears that a concentration of 1 part per million of water-soluble phosphorus (1 to 5 soil-water extract) will permit maximum growth of mustard. This level was maintained with approximately 500 pounds per acre of superphosphate on Norfolk fine sand, and 4,000 pounds per acre on Orangeburg fine sandy loam.

The data in table 1 show that the Orangeburg fine sandy loam has a much higher phosphate-absorbing power than either the Norfolk sand or the Norfolk fine sand. A better growth of vetch was obtained on the Norfolk soils without phosphates than on the Orangeburg soil with a 4,000-pound application of superphosphate per acre. A minimum of 0.5 parts per million of water soluble (1 to 5 soil-water extract) phosphorus was required to produce appreciable growth, while increased amounts produced an increase in growth until a level

of about 2 parts per million was reached. This level was higher than that required for mustard. The phosphorus content of the vetch and sorghum as shown in table 2, in general paralleled the water-soluble phosphorus in the soil. The highest phosphorus concentration attained in vetch was 0.75 percent  $P_2O_5$  when grown on Norfolk sand with 15.8 parts per million of water soluble phosphorus. The greatest concentration of water-soluble phosphorus in the Norfolk fine sand was approximately 8 parts per million while that for the Orangeburg fine sandy loam was 0.9 parts per million (table 1). But the phosphorus content of vetch from these two soils was less than that on the Norfolk sand (table 2).

TABLE 1.—Yield of mustard and vetch grown on three soil types as influenced by different amounts of water-soluble phosphorus

Crop and acre rate of superphosphate applied (pounds)	Water-soluble phosphorus in extracts			Oven-dry yield of crops from—		
	Norfolk sand	Norfolk fine sand	Orangeburg fine sandy loam	Norfolk sand	Norfolk fine sand	Orangeburg fine sandy loam
Mustard: <sup>1</sup>	<i>P. p. m.</i>	<i>P. p. m.</i>	<i>P. p. m.</i>	<i>Grams</i>	<i>Grams</i>	<i>Grams</i>
0.....	0.9	1.0	0.20	2.4	2.4	0.3
500.....	1.8	1.4	.28	2.4	3.5	1.1
1,000.....	2.3	1.8	.36	2.5	4.1	2.3
1,500.....	5.8	2.2	.39	2.5	4.3	3.7
2,000.....	7.8	2.9	.58	2.4	4.4	4.1
4,000.....	9.6	4.3	.85	2.6	4.0	4.5
8,000.....	17.2	10.3	1.90	2.4	4.5	4.2
Vetch: <sup>2</sup>						
0.....	.6	.4	( <sup>3</sup> )	1.8	1.5	.62
500.....	.9	.4	( <sup>3</sup> )	1.6	1.4	.41
1,000.....	1.2	.8	( <sup>3</sup> )	2.0	1.4	.35
1,500.....	2.2	1.6	.12	2.2	2.1	.51
2,000.....	3.2	2.7	.25	2.2	2.2	.90
4,000.....	5.8	3.1	.58	2.7	2.7	1.90
8,000.....	15.8	8.1	.90	2.1	2.5	2.40

<sup>1</sup> Superphosphate mixed with the soil 4 months fallow; planted to mustard; harvested after 60 days.

<sup>2</sup> Vetch followed mustard without additional superphosphate, but with additions of nitrogen and potash; harvested after 120 days.

<sup>3</sup> Trace.

TABLE 2.—Phosphorus content of vetch and sorghum grown on three soil types as influenced by rate of phosphate fertilization

Crop and acre rate of superphosphate applied (pounds)	Phosphorus content of crops from—			Crop and acre rate of superphosphate applied (pounds)	Phosphorus content of crops from—		
	Norfolk sand	Norfolk fine sand	Orangeburg fine sandy loam		Norfolk sand	Norfolk fine sand	Orangeburg fine sandy loam
Vetch: <sup>1</sup>	<i>Percent</i>	<i>Percent</i>	<i>Percent</i>	Sorghum: <sup>2</sup>	<i>Percent</i>	<i>Percent</i>	<i>Percent</i>
0.....	0.114	0.092	0.087	0.....	0.083	0.070	0.070
500.....	.109	.087	.092	500.....	.074	.083	.083
1,000.....	.114	.100	.087	1,000.....	.100	.079	.079
1,500.....	.118	.114	.087	1,500.....	.105	.092	.092
2,000.....	.170	.153	.096	2,000.....	.144	.100	.100
4,000.....	.249	.197	.118	4,000.....	.201	.105	.105
8,000.....	.328	.280	.157	8,000.....	.223	.157	.157

<sup>1</sup> Vetch followed mustard without additional superphosphate, but with additions of nitrogen and potash; harvested after 120 days.

<sup>2</sup> Sorghum followed vetch without additional superphosphate, but with additions of nitrogen and potash; harvested after 60 days.



FIGURE 2.—The effect of different amounts of phosphorus on the growth of vetch in: *A*, Norfolk sand; *B*, Norfolk fine sand, *C*, Orangeburg fine sandy loam. All cultures treated alike except for phosphorus additions. Nos 1 to 7 received 0, 500, 1,000, 1,500, 2,000, 4,000, and 8,000 pounds of superphosphate per acre, in numerical order.

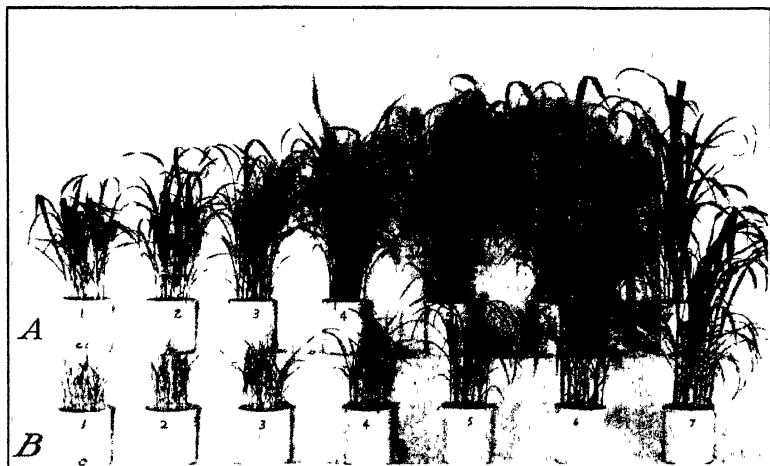


FIGURE 3.—The residual effect of varying amounts of phosphorus on the growth of sorghum in: *A*, Norfolk, fine sand; *B*, Orangeburg fine sandy loam. The sorghum represents the third crop following the application of phosphorus. Nos. 1 to 7 received 0, 500, 1,000, 1,500, 2,000, 4,000, and 8,000 pounds of superphosphate per acre, in numerical order.

The residual effects of the phosphate on the third crop are shown in figure 3, as compared with the initial effects in figure 4. These figures show that when sorghum was the initial crop, maximum growth occurred on the Norfolk fine sand at an application of 500 pounds of phosphate per acre, while additional increments on the Orangeburg soil up to 2,000 pounds produced additional increments of

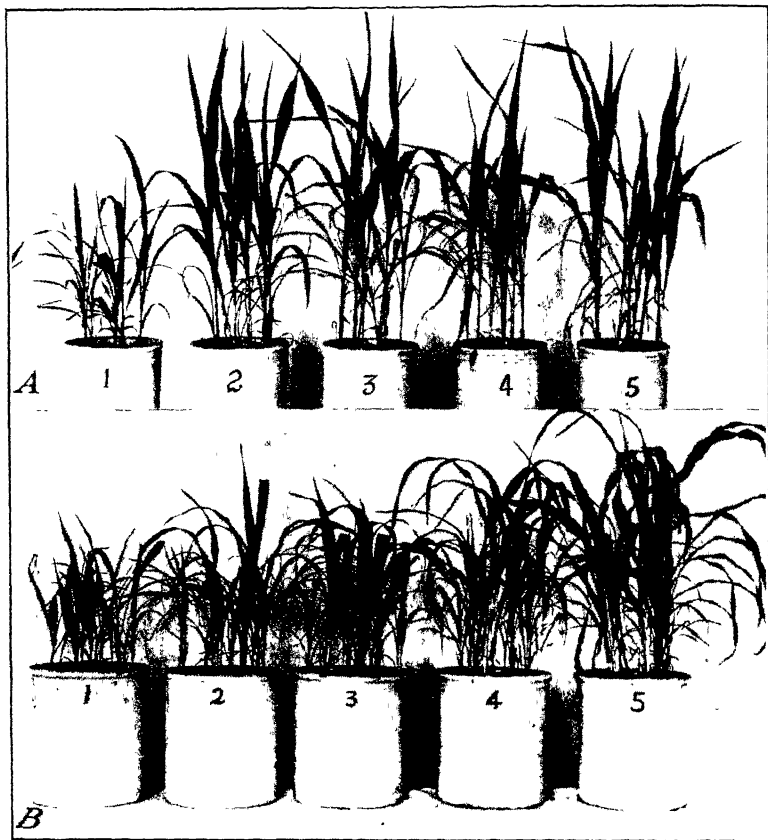


FIGURE 4.—The initial effect of varying amounts of phosphorus on the growth of sorghum in Norfolk fine sand (A), and Orangeburg fine sandy loam (B). All cultures treated alike except for phosphorus additions. Nos. 1 to 5 received 0, 500, 1,000, 1,500, and 2,000 pounds of superphosphate per acre, in numerical order.

growth. But when sorghum was the third crop, the original application of phosphate had become depleted or absorbed by the soils to such an extent that it required a 2,000-pound application for maximum growth in the Norfolk soil and 4,000 pounds in the Orangeburg soil.

The residual effects of the phosphate on the phosphate content of sorghum are also shown in table 2. Here, as in the case of the crop of vetch, the phosphorus content of the sorghum increased with the



content of water-soluble phosphorus in the soil. The results indicate that sorghum responds to phosphate fertilizers equally as well as vetch, but mustard apparently does not respond to phosphates as readily as do either of the other two crops.

#### DISCUSSION OF RESULTS

The response of the three crops to phosphate fertilization on these representative soils bears out expectations. The Orangeburg fine sandy loam would naturally fix the phosphates more completely than either the Norfolk sand or fine sand, because of the difference in fixing agents (silt and clay) even where lime is applied. This fixation was indicated by the difference in water-soluble phosphorus, growth of the plants, and phosphorus content of the plants.

Under the conditions of this study it appears that a concentration of water-soluble phosphorus (1 to 5 soil-water extract) equivalent to 0.5 part per million was required to secure appreciable crop growth. Increased concentration produced increased growth until a level of approximately 2 parts per million was reached. The phosphorus content was in general proportional to the water-soluble phosphorus in the soil but the general level was less in the Orangeburg soil than in the Norfolk soils.

The phosphorus content of the plants is comparable to that obtained by Becker, Neal, Shealy, and York (2) in wiregrass growing on areas of these soils. Five samples of wiregrass from the Orangeburg soils average 0.082 percent of phosphorus while eight samples from the unaffected areas averaged 0.133 percent. During the investigation of nutritional anemia, 35 samples of wiregrass from sands, fine sands, and loamy sands, representing sweeney-free areas, averaged 0.159 percent of phosphorus, according to Becker, Neal, Shealy, and York (2).

Because of the crowded condition of the roots in the small volume of soil used in this study the level of water-soluble phosphorus for maximum growth in the different soils would probably be different with different volumes of soil, but the response for the different soils would be comparable.

#### SUMMARY

A study was made in which varying amounts of superphosphate (0 to 8,000 pounds per acre) were added to Norfolk sand, Norfolk fine sand, and Orangeburg fine sandy loam. Mustard, vetch, and sorghum were used as test plants. Water-soluble phosphorus in soil extracts, yields of crops, and phosphorus content of the plants were determined.

The plants did not respond to a concentration of water-soluble phosphorus greater than 2 parts per million. A concentration of 0.5 part was necessary to secure appreciable growth of sorghum. The greatest response to phosphate addition was on the Orangeburg soil. The phosphorus content of the plants was proportional to the water-soluble phosphorus content of the soil. Because of the high fixing power of the Orangeburg soil for phosphorus, larger applications of the element were necessary for maximum results.

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# THE FUNCTION OF COPPER IN SOILS AND ITS RELATION TO THE AVAILABILITY OF IRON AND MANGANESE<sup>1</sup>

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## INTRODUCTION

Early experiments on the peat soils of North Carolina have shown a striking negative correlation between yields of corn and the accumulation of iron in the nodal tissues.<sup>2</sup> Studies of the oxidation-reduction potentials of these soils were undertaken in an attempt to associate the increase in the soluble iron content of the soil with factors favorable to reductiveness. It appears from the data obtained<sup>3</sup> that the soil potential is governed by an equilibrium between oxygen and the products of microbial decomposition of the organic matter of the soil.

While the exact nature of the oxidation-reduction reaction involved is not clearly apparent, it seems that it is chemical and spontaneous rather than biological. According to this concept, equilibrium potentials are dependent on a constantly replenished supply of oxygen and of the reductive product of microbial activity. The concentration of the latter appears to be extremely low, whereas the supply of oxygen is limited only by the factors governing aeration.

## THE EFFECT OF COPPER ON SOIL POTENTIALS

Early in the work on this problem it was considered probable that with adequate aeration, the concentration of the reductive component could be decreased by a catalyst that would accelerate the chemical reaction. With an abundant supply of oxygen, therefore, such a catalyst should raise the soil potential, whereas with deficient aeration the potential should be lowered, provided in the latter case the catalyst did not prevent microbial growth.

Evidence supporting the first of these assumptions is given in figure 1. The soil was a Dunbar fine sandy loam moderately high in organic matter. The copper sulphate was added to a suspension of

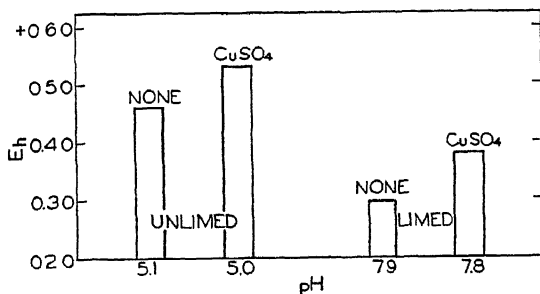


FIGURE 1.—The effect on oxidation-reduction potentials of copper sulphate added to limed and unlimed Dunbar fine sandy loam in aerated water suspension.

<sup>1</sup> Received for publication Oct. 22, 1935; issued April 1936. Published as paper no. 84 of the Journal Series of the North Carolina Agricultural Experiment Station.

<sup>2</sup> SHERWIN, M. E. SOIL TREATMENTS TO OVERCOME THE INJURIOUS EFFECTS OF TOXIC MATERIALS IN EASTERN NORTH CAROLINA SWAMP LAND. Jour. Elisha Mitchell Sci. Soc. 39: 43-48. 1923.

<sup>3</sup> WILLIS, L. G. SOME POTENTIAL CHANGES INDUCED BY LIMING SUSPENSIONS OF A PEAT SOIL. N. C. Agr. Expt. Sta. Tech. Bull. 47, 16 pp., illus. 1934.

the soil which was then shaken in the air for 24 hours and the potentials were determined in equilibrium with air.

The other assumption was verified by noting the potentials of deaerated suspensions of a peat soil without and with added copper sulphate. The results, shown in figure 2, suggest an activation of the micro-organisms in the suspension due to the addition of the copper sulphate, since the negative trend is most rapid with this treatment at the higher pH levels. In the more acid suspensions, however, there is a reversal of the sign of the slope of the potential/pH curve. Similar results have been observed in other work on soil potentials without the addition of copper sulphate and it has been assumed that these can be ascribed to increased activity of other metallic elements as well as copper that may be associated with greater solubilities at the lower pH levels. The type of potential/pH curve obtained with the suspensions containing copper is suggestive of

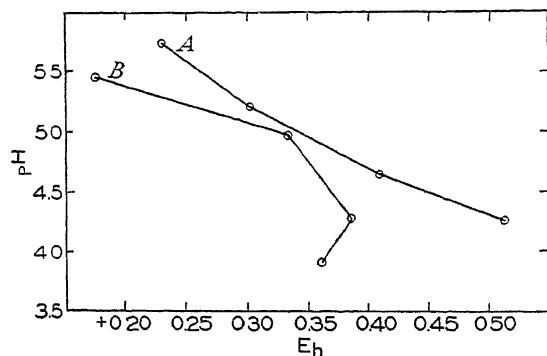


FIGURE 2—Oxidation-reduction potentials of peat-soil suspensions at different pH levels 9 days after the removal of oxygen. A, No copper sulphate added, B, copper sulphate added.

interesting possibilities for further study. Attention has been given recently to the linear character of soil potential/pH curves. Without attempting to expand the discussion beyond the scope of this article, it should be pointed out that this relation holds only where aeration, microbial activity, and other factors contributing to

the oxidation-reduction equilibrium can be treated as constant values.

Considerable emphasis has been given to the organic-matter content of the soils that have been used in oxidation-reduction studies.<sup>4</sup> These soils have been used largely for the purpose of determining fundamental relationships and trends. In soils of low organic-matter content it is not improbable that the reductive effects of microbial activity are developed only in the rhizosphere.<sup>5</sup> Potentials in such minutely localized zones would not be measurable but they may be expected to exhibit characteristics similar to those found in the more highly organic soils.

It has been suggested<sup>6</sup> that copper acts as an oxidizing catalyst. From the foregoing evidence it appears that this element activates oxidation-reduction reactions in the soil. The mechanism of this activation is not indicated by the results, but in consideration of the effectiveness of relatively small amounts of copper the function would appear to be catalytic.

<sup>4</sup> WILLIS, L. G. See footnote 3.

<sup>5</sup> STARKEY, R. L. SOME INFLUENCES OF THE DEVELOPMENT OF HIGHER PLANTS UPON THE MICROORGANISMS IN THE SOIL. IV. INFLUENCE OF PROXIMITY TO ROOTS ON ABUNDANCE AND ACTIVITY OF MICROORGANISMS. *Soil Sci.* 32: 367-393, 1931.

<sup>6</sup> (JACKS, G. V., and SCHERRATOFF, H. SOIL DEFICIENCIES AND PLANT DISEASES. Imp. Bur. Soil Sci. Harpenden) *Tech. Commun.* 31, 1934.

## EVIDENCES OF COPPER-IRON ANTAGONISM

Much of the evidence to be reported consists of a series of tests of a hypothesis of an antagonistic effect of copper relative to iron. Originally it was assumed that this could be attributed to a direct oxidation of the iron in the soil. Apparently the reactions involved are more complex than this, yet the hypothesis has been of considerable value as a basis for experimentation.

The first experimental test of the hypothesis, in which it was shown that the iron content of corn plants grown on a peat soil could be reduced by applications of copper sulphate, has already been published.<sup>7</sup> A statement of the hypothesis was purposely avoided in that publication pending more supporting evidence. This is furnished by the following observations and data which are presented in sequence.

## RESULTS WITH CORN

A field-plot experiment with corn (*Zea mays*) on a peat soil was designed to incorporate all the soil treatments that prior experiments had shown to be favorable. The first crop was virtually a failure on all plots. Between seasons some of the soil was put into pots in the greenhouse so that tests could be made of various supplementary soil treatments. Of these, only copper sulphate gave any evidence of a definite effect. With this compound the seedling corn made a rapid early growth, but the leaves soon became chlorotic between the veins.

An application of a 1-percent solution of ferrous sulphate rubbed lightly on spots on these chlorotic leaves caused a number of them to turn green (fig. 3). Spots rubbed in a similar manner with distilled water did not change, nor was there any effect noted where manganous sulphate was applied alone or with the ferrous sulphate.

It was evident that the application of copper sulphate to the soil produced an iron-deficiency chlorosis. Since the copper sulphate was applied in a thin layer midway of the depth of the soil in the pots, it does not appear reasonable to conclude that the copper modified the properties of the soil mass. These results raise the question whether the copper was effective in the soil or in the plants and also whether the manganese had an effect similar to that of copper.

## RESULTS WITH COTTON

Later a series of solution culture experiments was started with cotton (*Gossypium hirsutum*). Copper, zinc, manganese, and boron were added uniformly from a stock solution to all the cultures in amounts supplying 0.1 mg of each of the elements per liter of solution. Iron was supplied as ferric citrate. In the first attempt a severe iron-deficiency chlorosis developed in the fifth leaf of the plants. The work was repeated with the omission of the copper-zinc-manganese-boron solution until the plants were 10 inches high. No chlorosis developed until the plants were over 2 feet high. Then the terminal leaves became chlorotic in the cultures supplying nitrogen as sodium nitrate. The use of the copper-zinc-manganese-boron solution was discontinued and the affected leaves became green within 4 days.

<sup>7</sup> WILLIS, L. G., and PILAND, J. R. THE INFLUENCE OF COPPER SULPHATE ON IRON ABSORPTION BY CORN PLANTS. Soil Sci 37 79-83, illus 1934

Obviously no question of the oxidation of iron is involved in this case. While the use of the four-element stock solution was a matter of convenience, it was assumed that the copper salt was the significant component. Attention was therefore centered on this element with-

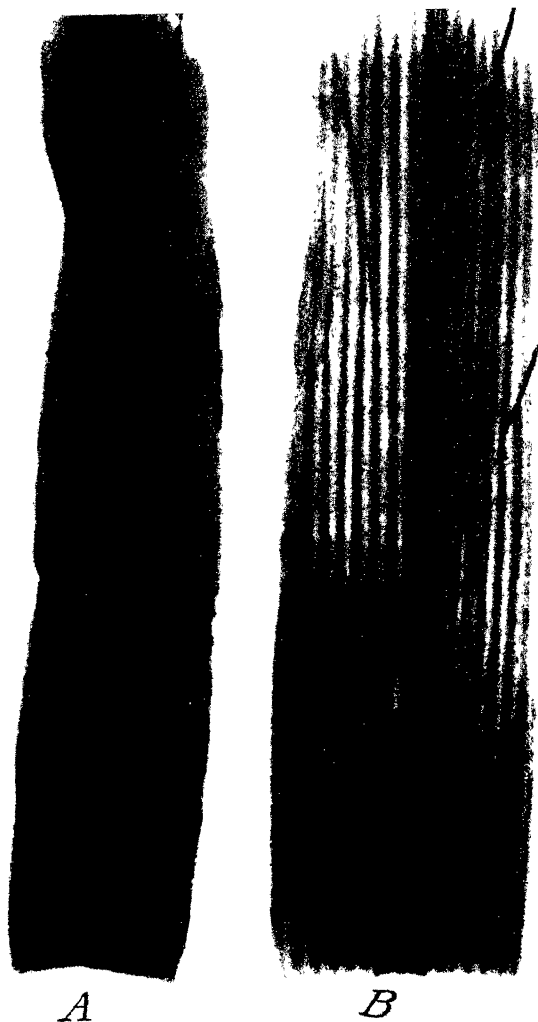


FIGURE 3—Iron-deficiency chlorosis produced in corn leaves by the addition of copper sulphate to a peat soil. *A*, leaf of plant grown in soil without treatment; *B*, leaf of plant grown in soil to which copper sulphate was added, a green area produced by the external application of ferrous sulphate solution being shown at *a*.

out prejudice toward a possibility of a similar effect of the others. Possibly the copper effected the decomposition of the citrate radical, or the inactivation of the iron may have taken place within the plant. Another possibility carries the implication that the iron of ferric

citrate must be reduced before it becomes assimilable by plants and that copper prevents this reduction. This point must be verified before it can be given serious consideration.

The delayed onset of the iron-deficiency chlorosis in the solutions supplied with sodium nitrate occurred at the time that blossom buds were beginning to form. Whether or not the effect has any relation to physiological changes developing at this period of growth is debatable, but the difficulties of the translocation of relatively insoluble elements through long stem growth may have a bearing on the effect of copper.

#### IDENTIFICATION OF COPPER EFFECT

In an effort to verify the assumption that the effect of the stock solution could be attributed to copper, some cotton plants that had been grown in solution cultures without copper or manganese were used as indicators. Iron had been supplied as ferric citrate for the early growth of the plants, but later inclusion of iron was discontinued until the plants showed a definite chlorosis symptomatic of an iron deficiency.

The stems were split from the base for a distance of about 2 inches, leaving approximately one-half of the lateral roots on each segment. Nutrient solutions were put into 500-ml Erlenmeyer flasks which were bound together in pairs so that the tops were in contact and at the same level. The plants were transferred to these flasks, one segment being inserted into each of the paired flasks.

The nutrient solution used had the following composition in grams per liter: Monopotassium phosphate, 0.0892; dipotassium phosphate, 0.1140; sodium chloride, 0.0262; potassium sulphate, 0.1550; potassium chloride, 0.1326; magnesium sulphate  $-7 \text{ H}_2\text{O}$ , 0.1363; calcium chloride  $-2 \text{ H}_2\text{O}$ , 0.0992; sodium nitrate, 0.1900.

In addition to this, ferric citrate and copper sulphate were added as shown in table 1.

Since it has frequently been observed that symptoms of deficiencies of some of the minor essential elements are not correctable in older leaves, all of these were removed at the time the plants were transferred to the flasks.

Within a week it was unmistakably apparent that the leaves of plants in the A series were becoming normally green. In the C series no evidence of recovery was observed, while the plants in the B series were intermediate in color in the early stages of growth. Later the differences between the A and B series became indistinguishable. The contrast in color of leaves from the A and C series is indicated in figure 4. Identification of the iron deficiency was made by spot treatments with ferrous sulphate on the surface of the leaves from the C series (fig. 4, B,  $\alpha$ ). It appears from this evidence that the effect of copper is largely external to the plant, but the delayed onset of iron deficiency in the preceding solution-culture experiment suggests the immobilization of iron within the plant as an additional factor.

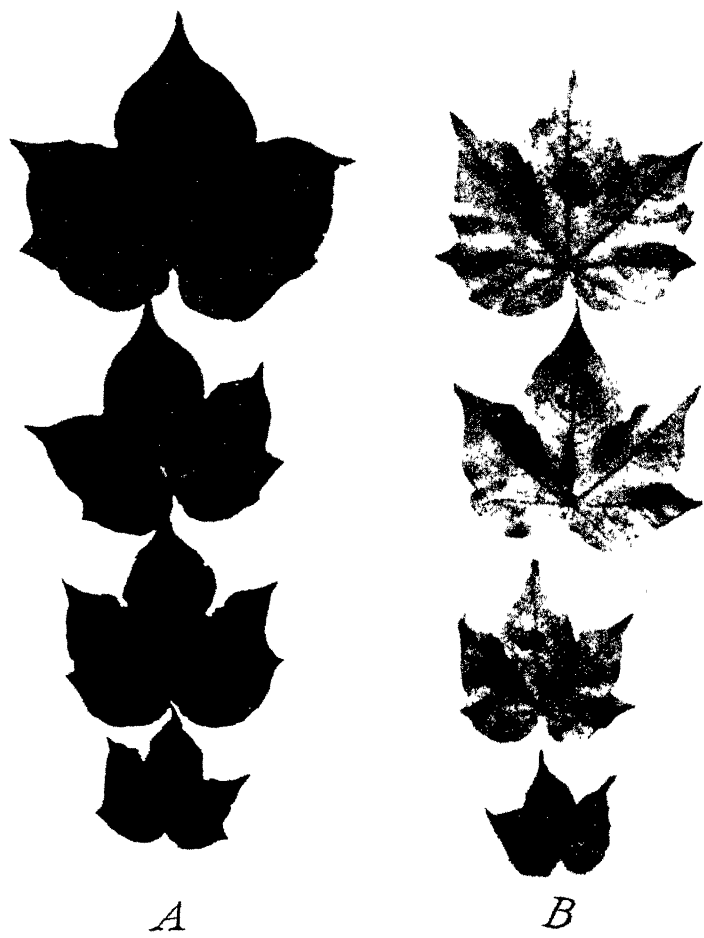


FIGURE 4.—Iron-deficiency chlorosis in cotton leaves resulting from the addition of copper sulphate to culture solutions: *A*, Solutions containing ferric citrate, series *A*, *B*, solutions containing ferric citrate and copper sulphate, series *C*, a green area produced by the external application of ferrous sulphate solution being shown at *a*.

TABLE 1.—Iron and copper additions made to nutrient solutions used in identifying the effect of copper upon cotton plants grown therein

Series	Flask no.	Addition
A.....	(1).....	Ferric citrate
	(2).....	Nothing.
B.....	(1).....	Ferric citrate.
	(2).....	Copper sulphate.
C.....	(1).....	Ferric citrate and copper sulphate.
	(2).....	Nothing.



## EVIDENCES OF COPPER-MANGANESE ANTAGONISM

Another case bearing on the effect of copper has been given some attention. Fairly extensive areas of soil in the upper Coastal Plain have failed occasionally to produce cotton satisfactorily. Some of this soil was shipped to Raleigh, N. C., and used for pot experiments. The pH value of the soil was 4.9. In the first series of pots it was found that lime was without effect and that fertilizer reduced the growth of the cotton plants. Some benefit was noted where copper sulphate was added to the soil receiving lime and fertilizer with lime.

In a second series of pots, fertilized with ammonium phosphate, ammonium nitrate, and potassium chloride supplemented with varying amounts of calcium sulphate, the best plants were produced on the soils receiving the minimum amount of calcium sulphate. A qualitative analysis of plants from these pots gave strong positive tests for manganese.

Samples of soil and cotton stalks were then obtained in midwinter from fields showing the characteristic damage in varying degrees. The trouble could be correlated with the manganese content of the stalks very closely, as is shown in table 2.

TABLE 2.—*Severity of symptoms in abnormally developing cotton plants as associated with the soil pH and the manganese and iron content (parts per million) of the stalks*

Symptoms	pH of soil	Manganese content of plants	Iron content of plants	Symptoms	pH of soil	Manganese content of plants	Iron content of plants
Slight.....	4.8	20	44	Moderate.....	5.5	15	48
Severe.....	4.6	52	48	Do.....	5.5	23	62
Slight.....	4.9	20	25	Do.....	5.5	29	33
Severe.....	4.6	53	38	Severe.....	5.5	30	34
None.....	5.7	10	20	None.....	-----	11	23

No significant correlation was found between the poor growth of cotton and either the pH of the soil or the iron content of the stalks.

The soils showing the severest symptoms gave definite qualitative tests for manganese in the water extracts from 5-g samples and stronger tests with the sodium chloride extract.

Further investigation established the fact that the soils involved were invariably found on the second bottom of rivers draining parts of North Carolina and Virginia where soils originating from manganeseiferous basic igneous rocks are common. It was concluded from the evidence that the manganese found in these soils existed in the bivalent form in the exchange complex and that it was not readily oxidizable to insoluble forms.

It has been found elsewhere in North Carolina that manganese becomes insoluble to the point of deficiency only in soils naturally high in organic matter.<sup>8</sup> A third series of pots was therefore started in which organic matter in the form of finely powdered peat was added. No improvement was observed in the cotton grown on this soil. In fact, the plants grown where lime was added showed abnormal structural deformities characterized by deeply lobed leaves and no

<sup>8</sup> WILLIS, L. G., and MANN, H. B. MANGANESE AS A FERTILIZER. Amer. Fert. 72 (1) [21]-24, illus, 1930.

elongation of the internodes. After 6 weeks it became evident that no further development was being made. At that time 0.1 g of copper sulphate was added in solution to the surface of the soil in each pot. The plants responded noticeably within 2 days and in 2 weeks had become normal in all respects (fig. 5). In no prior case



FIGURE 5.—Maximum growth of cotton plants in soil containing an excess of soluble manganese, limed (A) and unlimed (B), and condition of the same two plants respectively (C and D) 2 weeks after the addition of copper sulphate.

had any healthy lateral roots developed on any plants. Within 2 weeks after the addition of the copper sulphate, however, the growth of lateral roots had extended throughout the soil in all pots, although it is hardly probable that the copper sulphate had penetrated deeply into the soil.

A fourth attempt at the solution of this problem was made, but in this case cotton grew normally in all the pots irrespective of the treatments. The soil had been stored dry in the greenhouse while the work was in progress and this seems to have eliminated the injurious factor. Inquiries regarding the trouble in the field have brought the unanimous opinion from farmers that crops are damaged severely only in wet seasons.

Although the evidence is not conclusive, there is some support for the assumption that the maintenance of manganese in a state of oxidation in field soils is dependent on the presence of a catalyst such as copper.

#### SUGGESTED LIMITATIONS TO THE USE OF COPPER SULPHATE IN SOILS

The foregoing results support the conclusion that copper sulphate as it is generally used in soils serves largely as a soil amendment for the correction of conditions under which the excessive concentrations of soluble iron and possibly manganese exist. It is not impossible, however, that there are occasional instances of soils in which copper is deficient in an absolute sense and where this element may be needed as a direct plant nutrient.

It would seem that copper sulphate would be most beneficial when applied to aerated soils having a high content of decomposable organic matter. Where the oxygen supply is limited the effect of copper may be injurious. In well-aerated soils low in total iron and organic matter, copper may cause an iron deficiency except at low pH values. Until these factors have been more completely investigated the indiscriminate application of copper sulphate to soils would appear to be inadvisable.

#### SUMMARY

Data already published indicate that the oxidation-reduction potential of soils is governed by an equilibrium between oxygen and a chemically active reducing compound which is the product of microbial activity.

It was assumed that this equilibrium could be modified by a catalyst such as copper added to the soil and that this would be reflected in the solubility of the iron compounds of the soil. In preliminary tests it was found that copper sulphate increased the potentials of an aerated soil suspension but decreased the potentials when air was excluded. The significance of these effects was indicated by published evidence that copper sulphate decreases the iron content of corn plants grown on a peat soil.

Corn grown on an unproductive peat soil became chlorotic when copper sulphate was added to the soil. The chlorosis was identified as being due to an iron deficiency when ferrous sulphate applied externally to the leaves caused the development of a green color. The application of manganese sulphate alone or in combination with ferrous sulphate had no visible effect.

Cotton plants grown in solution cultures containing either nitrate or ammoniacal nitrogen and ferric citrate, copper, manganese and zinc sulphates, and boric acid developed an iron-deficiency chlorosis in their early growth. In a repetition of the work with the addition

of manganese, zinc, copper, and boron delayed, a chlorosis developed in the plants receiving nitrate nitrogen when they were 2 feet high. Following the elimination of the four elements from the solution the affected plants rapidly became green.

An experiment in which the roots of iron-deficient cotton plants were divided between two solution cultures has given evidence that copper sulphate will produce an iron-deficiency chlorosis. It appears that this effect is due largely to reactions external to the plant, but there is also evidence of immobilization of iron within the plant under the influence of copper.

Further evidence in support of the assumption that copper serves as a catalyst of oxidation-reduction reactions is presented by a preliminary study of a field soil problem. The soil contained abnormally large quantities of water-soluble and exchangeable manganese. Beneficial effects were observed following applications of copper sulphate or a prolonged drying of the soil.

Copper sulphate serves as a soil amendment, decreasing the availability of iron and possibly of manganese. The effect may be favorable or not depending on the oxidation intensity and the iron and manganese content of the soil.

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## THE EFFECT OF DIFFERENT COLLOIDAL SOIL MATERIALS ON THE TOXICITY OF CALCIUM ARSENATE TO MILLET<sup>1</sup>

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### INTRODUCTION

At one time it was generally assumed that the arsenical and other poisonous sprays used for controlling insect pests had no injurious effect on soil productivity. This may have been true years ago, when the quantities applied were usually small, but it apparently does not hold for the much heavier applications that are frequently used at the present time. Albert, Cooper, Paden, and others at the South Carolina Agricultural Experiment Station (1, 2, 3, 7, 8)<sup>2</sup> have shown, for instance, that the comparatively large applications of calcium arsenate used to control the boll weevil may injure the soil for subsequent crops. According to their observations, sandy soils are more susceptible to injury from such applications than heavier soils and gray soils are more susceptible than red soils. Dratschew (9) has shown that a clay may adsorb 1.04 percent of its weight in arsenate ions from a 0.1-normal solution, but he has not studied the effect of such adsorption on plant growth.

It seemed from previous work that the resistance of soils to arsenic injury should be determined by the kind of colloid in the soil as well as by the amount. It also seemed probable that the various effects of the colloids on calcium arsenate would be correlated with the silica-sesquioxide ratios of the materials, since the fixation of phosphate ions is so correlated. Accordingly, experiments were conducted with 36 soils and subsoils to compare the effects of the colloids on the toxicity of calcium arsenate.

### METHODS

The method employed was similar to that used in a previous study of the effects of soil colloids on the efficiency of superphosphate (10). Foxtail millet (*Setaria italica*) was grown in different soil-sand mixtures, each of which contained sufficient soil to supply 1 percent of colloidal material to the mixture. Increasing applications of calcium arsenate were made to each mixture and the oven-dry weights of the crops determined. By plotting yields against calcium arsenate applications, the quantity of arsenic pentoxide required to reduce the yield by one-half could be determined for each soil-sand mixture. These quantities, minus the quantity of arsenic reducing the yield one-half in pure quartz sand, gave figures for comparing the effects of the different colloids on arsenic toxicity.

<sup>1</sup> Received for publication Oct. 21, 1935, issued April 1936.

<sup>2</sup> Reference is made by number (italic) to Literature Cited, p. 490.

It will be seen from the curves in figure 1 that similar comparative values would have been obtained if the soils or colloids had been compared on the basis of the arsenic required to reduce the yield by one-fourth, three-fourths, or by any specified amount. But the minimum lethal dose could not well be used for comparison, since this value is not sharply defined.

It will be noted that in this experimental procedure the material added to the sand is not the pure colloid but the whole soil in an amount inversely proportional to its colloid content. This procedure, of course, gives a comparison of the colloids only in case the noncolloidal soil material is without effect on the toxicity of calcium arsenate. This assumption is probably substantially correct, since many studies indicate that the reactions of soils to various salts are confined almost exclusively to the colloidal material. Thus, in the following pages when the effect of the soil colloids is discussed

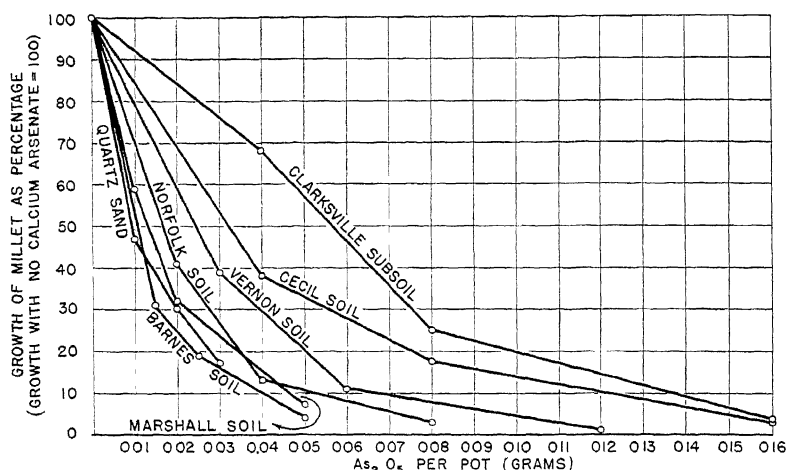


FIGURE 1.—Effects of increasing quantities of calcium arsenate on the growth of millet in soil-quartz sand mixtures.

it is understood that an assumption is involved which seems to be well justified by recent soil investigation.

It may seem that any assumption at all could have been avoided by using the isolated colloidal material. But, aside from its difficulties, this procedure would have led to known errors. Part of the colloid cannot be extracted from certain soils by present methods, and, still more important, the properties of the colloid are altered by the extraction process. Although the alteration may have slight effect on certain laboratory determinations, it may affect plant growth markedly if the freshly extracted colloid is used, as has previously been shown (10).

Except for the phosphate application, all pots received the same basic fertilizer; namely, 0.93 g of potassium nitrate, 0.33 g of ammonium sulphate, 0.0185 g of ferric tartrate, 0.42 g of  $MgCl_2 \cdot 6H_2O$ , and 0.0015 g of  $MnSO_4 \cdot 2H_2O$  per pot. The phosphate, supplied as superphosphate, varied from 0.2 g of  $P_2O_5$  per pot to 0.6 g of  $P_2O_5$ , according to the kind of colloidal material present. In some cases the amount of  $P_2O_5$  necessary for maximum growth could be roughly

calculated from the data of previous experiments; in other cases it was estimated from the silica-sesquioxide ratio of the colloid.

The tricalcium arsenate used was the "c. p. analyzed reagent" of a well-known manufacturer of analytical chemicals. This material and the superphosphate were thoroughly mixed with the sand. The other salts were applied in solution just prior to planting. Glazed earthenware pots holding 5,000 g of quartz sand were used as containers. They were kept in a greenhouse, and their order was shifted every day or two. The moisture content of the sand or sand-soil mixture was maintained at 15 percent. Ten millet plants per pot were grown for 25 to 40 days according to the time of year, ordinarily when the heads were about to appear. The differently treated pots were replicated three times in each experiment.

The soils used in this study were parts of samples utilized in former investigations of soil colloids. Data regarding the samples with laboratory numbers below 500 are given in two bulletins of the United States Department of Agriculture (11, 14), and descriptions of the samples with higher laboratory numbers are given in two others (6, 13). In some cases the colloid content of the soil was determined by adsorption of water vapor, in other cases by mechanical analysis.

#### CRITICAL ARSENIC VALUES FOR DIFFERENT SOIL COLLOIDS

A few preliminary experiments were conducted to get an approximate idea of the toxicity of calcium arsenate to millet under the experimental conditions. The soil colloids were then compared in 11 experiments. These were conducted at different times of the year under various conditions of temperature and sunlight. Growth varied markedly, of course, in some of the experiments, and it seemed possible that the toxicity of arsenic in the plant might vary with these conditions to such an extent that the results of the different experiments would not be comparable. Such, however, was evidently not the case. Each experiment included a standard series of pots in which calcium arsenate was added to pure quartz sand. Growth in this series varied markedly with the season in which the experiment was conducted, but the quantity of arsenic required to reduce the yield one-half was practically the same for all experiments, irrespective of the season. In 9 of the 11 experiments the critical quantity of arsenic for pure quartz sand varied only between 0.008 and 0.013 g of  $\text{As}_2\text{O}_5$  per pot, with an average value of 0.011 g having a probable error of  $\pm 0.00036$  g. In 1 experiment the critical quantity was not closely determined but was obviously less than 0.016 g, and in the remaining experiments the exceptional value of 0.021 g was obtained.

The results of the quartz sand series provided a blank for the experiment which was to be subtracted from results yielded by the soil-sand mixtures to obtain the effects of the colloids on arsenic toxicity. In view of the constancy of the results of this series it was felt that the average figure was as accurate a blank for any experiment as the individual value obtained for that experiment. Consequently 0.011 g of  $\text{As}_2\text{O}_5$  was used as a blank for all experiments.

The detailed results of the experiments in which critical arsenic values were obtained for 36 soil colloids are shown in tables 1 and 2. The results of the pure quartz sand series are not reported in these tables, since with the use of the average value for this blank series the individual results are of no interest.

TABLE 1.—*Effects of different soils on the toxicity of calcium arsenate*

[All soils added in sufficient quantity to supply 1 percent colloid]

## EXPERIMENT 5 (FEB 15 TO MAR. 18)

Laboratory no.	Type of soil	Depth	P <sub>2</sub> O <sub>5</sub> applied per pot	Cal-cium arse-nate added per pot, ex-pressed as As <sub>2</sub> O <sub>3</sub>	Oven-dry yield of plants per pot				As <sub>2</sub> O <sub>3</sub> re-quired to re-duce yield one-half	As <sub>2</sub> O <sub>3</sub> reduc-ing yield one-half minus the blank of 0.011 g for quartz sand alone
					Series A	Series B	Series C	Average		
		Inches	Gram	Gram	Grams	Grams	Grams	Grams	Gram	Gram
10305	Barnes loam.....	0-9	0.12	0	3.37	3.40	3.83	3.53	0.011	0.000
				.015	1.16	.83	1.35	1.11		
				.025	.55	.56	.94	.68		
				.050	.22	.12	.10	.15		
10341	Miami silt loam.....	0-1½	.15	0	3.31	3.31	3.28	3.30	.018	.007
				.020	1.74	1.53	1.32	1.53		
				.040	1.02	.63	.85	.83		
				.080	.15	.21	.25	.20		
4439	Davidson loam.....	0-9	.20	0	3.92	3.11	3.54	3.52	.027	.016
				.04	1.08	.95	.79	.94		
				.08	.32	.29	.30	.30		
				.16	.08	.09	.08	.08		
5891	Niipe clay.....	0-12	.50	0	1.36	1.33	1.37	1.35	.103	.092
				.15	.30	.37	.39	.35		
				.25	.04	.04	.04	.04		
				.40	.04	.05	.05	.05		

## EXPERIMENT 6 (MAR 24 TO APR 24)

10082	Carrington loam.....	0-3	0.15	0	6.45	5.79	5.43	5.89	0.018	0.007
				.01	-----	4.61	3.96	4.28		
				.02	2.95	-----	2.81	2.88		
				.05	.57	.49	.58	.55		
195	Clarksville silt loam.....	0-10	.20	0	4.73	3.64	3.56	3.98	.032	.021
				.02	3.74	2.58	2.55	2.96		
				.04	1.39	1.27	1.50	1.39		
				.08	.33	.39	.36	.36		
196	do.....	10-36	.40	0	4.39	6.40	5.72	5.50	.058	.047
				.04	3.84	3.60	3.72	3.72		
				.08	1.59	1.40	1.15	1.38		
				.16	.21	.18	.15	.18		
9415	Cecil clay loam.....	0-6	.40	0	3.84	4.37	-----	4.11	.031	.020
				.04	1.44	1.60	1.69	1.58		
				.08	.87	.02	.57	.69		
				.16	-----	.13	.14	.14		
6678	Kirvin fine sandy loam.....	0-12	.35	0	5.63	5.49	5.43	5.52	.056	.045
				.02	4.73	5.10	4.99	4.94		
				.04	3.78	3.74	3.80	3.77		
				.08	1.36	.99	1.43	1.26		

## EXPERIMENT 10 (SEPT. 16 TO OCT. 13)

183	Norfolk fine sandy loam...	0-8	0.40	0	3.47	3.67	4.26	3.80	0.017	0.006
				.02	1.74	1.63	1.24	1.54		
				.08	.62	.50	.34	.49		
				.10	-----	-----	.11	.11		
184	do.....	12-36	.50	0	3.50	2.87	2.50	2.96	.034	.023
				.04	1.41	-----	1.03	1.22		
				.08	.34	-----	-----	.34		
				.16	.07	.07	.07	.07		
6718	Vernon fine sandy loam...	0-3	.30	0	3.60	3.74	3.50	3.61	.025	.014
				.03	1.24	1.83	1.10	1.39		
				.06	.42	.45	.31	.39		
				.12	.04	.04	.04	.04		
6719	do.....	3-10	.40	0	5.26	4.85	4.68	4.93	.028	.017
				.04	1.33	1.36	1.26	1.32		
				.08	.23	.22	.27	.24		
				.16	.03	.03	.03	.03		
6720	do.....	10-27	.50	0	5.13	4.54	4.55	4.74	.033	.022
				.04	1.83	-----	1.89	1.86		
				.08	.42	.61	.43	.49		
				.16	.05	.05	.05	.05		



TABLE 1.—*Effects of different soils on the toxicity of calcium arsenate*—Continued

EXPERIMENT 12 (NOV. 1 TO DEC. 11)

Laboratory no	Type of soil	Depth	P <sub>2</sub> O <sub>5</sub> applied per pot	Cal-cium arse-nate added per pot, ex-pressed as As <sub>2</sub> O <sub>5</sub>	Oven-dry yield of plants per pot				As <sub>2</sub> O <sub>5</sub> re-quired to re-duce yield one-half	As <sub>2</sub> O <sub>5</sub> reduc-ing yield one-half minu the blank of 0.011 g for quartz sand alone
					Series A	Series B	Series C	Average		
		<i>Inches</i>	<i>Gram</i>	<i>Gram</i>	<i>Grams</i>	<i>Grams</i>	<i>Grams</i>	<i>Grams</i>	<i>Gram</i>	<i>Gram</i>
B171	Marshall silt loam.....	0-13	0.20	0 .01 .02 .03 0	3.40 2.18 1.82 1.13 2.59	2.69 2.40 1.38 .83 2.77	2.49 2.37 1.85 .60 3.06	2.86 2.32 1.68 .85 2.81	0.023	0.012
B172	.....do.....	13-24	.25	0 .01 .02 .03 0	3.09 2.31 1.74 1.86 .89	2.50 1.75 1.30 1.90 1.00	2.59 2.18 1.40 1.14 .98	2.73 2.08 1.48 1.63 .96	.031	.020
6096	Houston black clay.....	0-3	.20	0 .01 .02 .04 0	.38 14 2.62 1.40 .60	.64 .14 2.54 1.49 .66	.74 .11 2.38 1.28 .57	.59 .13 2.51 1.39 .61	.014	.003
6097	.....do.....	14-20	.25	0 .01 .02 .04 0	.27 1.89 .60 .27 1.59	.18 2.58 .66 .18 1.77	.28 2.19 .57 .28 .95	.24 2.22 .61 .64 1.44	.012	.001
6098	.....do.....	24-36	.30	0 .01 .02 .04 0	.77 1.31 .33	.55 1.24 .25	.67 1.19 .23	.66 1.25 .27	.014	.003

EXPERIMENT 14 (MAR 7 TO APR. 6)

299	Chester loam.....	0-8	0.36	0 .02 .04 .06 0	5.63 4.17 3.09 1.98 3.18	4.85 3.88 2.92 1.86 3.10	5.66 4.19 3.08 1.99 3.50	5.38 4.08 3.03 1.94 3.26	0.046	0.035
300	.....do.....	8-32	.50	0 .02 .04 .06 0	3.06 2.39 1.20 6.12 4.79	3.10 2.31 1.44 5.94 4.60	2.84 2.32 1.29 5.47 4.70	3.00 2.34 1.31 5.64 4.70	.053	.042
391	Hagerstown loam.....	0-8	.36	0 .02 .04 .06 0	3.74 4.84 4.91 4.06 3.12	3.32 4.91 3.96 2.79 2.04	3.53 4.84 4.77 3.98 2.63	3.53 4.84 4.84 3.98 2.85	0.079	.068
181	Ontario loam.....	0-12	.36	0 .02 .04 .06 0	4.56 3.40 2.01 1.31	4.28 2.94 2.09 1.24	4.18 2.88 2.17 1.19	4.34 3.07 2.09 1.25	.051	.040
182	.....do.....	12-22	.50	0 .03 .06 .09 0	3.40 2.01 1.31	2.94 2.09 1.24	2.88 2.17 1.19	3.07 2.09 1.25	.057	.046

EXPERIMENT 15 (APR. 17 TO MAY 12)

297	Manor loam.....	0-7	0.30	0 .02 .04 .06 0	4.91 3.52 1.87 .96 4.41	5.49 3.72 1.83 1.13 4.71	5.25 3.50 1.48 1.03 4.58	5.22 3.58 1.73 1.04 4.57	0.031	0.020
298	.....do.....	7-20	.40	0 .02 .04 .06 0	3.46 1.82 .83 1.77 1.88	3.28 1.57 .90 1.67 2.03	2.93 1.39 .83 1.96 1.40	3.22 1.59 .89 1.80 1.77	.031	.020
10541	Caribou loam.....	34-2	.30	0 .02 .04 .06 0	1.30 1.03 .96 1.03 1.78	1.07 1.03 .97 1.30 1.45	1.96 1.12 .96 2.64 1.48	1.96 1.15 .96 1.66 1.56	.064	.053
10543	.....do.....	4-6	.40	0 .02 .04 .06 0	.72 6.27 3.56 1.74 .37	.39 5.77 3.83 1.86 .58	.61 6.95 3.32 2.01 .49	.57 6.33 3.57 1.87 .48	.051	.040
305	Stockton clay adobe.....	0-38	.20	0 .01 .02 .04 0	3.56 1.74 .37	3.83 1.86 .58	3.32 2.01 .49	3.57 1.87 .48	.012	.001

TABLE 1.—*Effects of different soils on the toxicity of calcium arsenate*—Continued

EXPERIMENT 77 (AUG. 18 TO SEPT. 12)

Laboratory no	Type of soil	Depth	P <sub>2</sub> O <sub>5</sub> applied per pot	Calcium arsenate added per pot, ex- pressed as As <sub>2</sub> O <sub>5</sub>	Oven-dry yield of plants per pot				As <sub>2</sub> O <sub>5</sub> re- quired to re- duce yield one- half	As <sub>2</sub> O <sub>5</sub> reduc- ing yield one- half minus the blank of 0.011 g for quartz sand alone
					Series A	Series B	Series C	Aver- age		
		Inches	Gram	Gram	Grams	Grams	Grams	Gram	Gram	
512	Sharkey clay.....	0- 4	0 20	0	4 60	3 77	4 28	4 22	0 031	0 020
				.01	3 60	3 33	3 34	3 42		
				.02	2 99	3.25	2.13	2 79		
				.04	1 61	1 63	1 54	1 59		
B3632	Houston black clay.....	1-15	25	0	4.37	4.54	4.36	4.42	.018	.007
				.01	3 02	3 21	2.84	3 02		
				.02	2 16	1.99	1.85	2.00		
				.04	.48	.56	.57	.54		
189	Wabash silt loam.....	0-15	20	0	3 95	4 44	-----	4 20	.026	.015
				.01	3 80	3.55	-----	3 68		
				.03	1 64	-----	-----	1 64		
				0	4 27	4.15	3 73	4 05		
190	-----do-----	15-36	20	.01	3 42	2 78	2 91	3 04	.024	.013
				.02	1.98	2 38	2.42	2 26		
				.04	.95	1 09	1.08	1 04		
				0	3 95	3 75	3.99	3 90		
9804	Columbiana clay.....	0-10	.50	.02	3 58	3.24	3 72	3.51	.093	.082
				.04	3 21	3 57	2 99	3 26		
				.08	2.15	2 19	2.44	2 26		

TABLE 2 — *Effects of increasing quantities of soil in soil-sand mixtures on the toxicity of calcium arsenate*

EXPERIMENT 7 (APR. 21 TO MAY 17)

Laboratory no	Kind of soil mixed with quartz sand and quantity in terms of colloid content per pot	Depth	P <sub>2</sub> O <sub>5</sub> applied per pot	Calcium arsenate added per pot, expressed as As <sub>2</sub> O <sub>5</sub>	Oven-dry yield of plants per pot				As <sub>2</sub> O <sub>5</sub> re-quired to reduce yield one-half	As <sub>2</sub> O <sub>5</sub> reduc-ing yield one-half minus the blank of 0.011 g for quartz sand alone
					Series A	Series B	Series C	Average		
		Inches	Gram	Gram	Grams	Grams	Grams	Grams	Gram	Gram
5028	Nacogdoches very fine sandy loam, 25 g.-----	0-8	0.30	0 .01 .02 .04 0	4 36 3 28 2 47 70	4 70 3 54 2 20 68	4 01 3 13 2 26 .67	4 36 3 32 2 31 .68	0.022	0.011
5028	Nacogdoches very fine sand loam, 50 g.-----	0-8	.50	0 .02 .04 .08 0	5 19 3 62 2 40 .49	5 27 3 63 2 39 47	4 78 3 25 1 57 34	5 08 3 50 2 12 .43	.035	.024
5028	-----do-----	0-8	.75	0 .02 .04 .08 0	5 10 3 74 2 80 .72	4 90 3 34 2 65 .71	4 76 3 43 2 19 .95	4 92 3 50 2 18 .79	.040	.029
5028	Nacogdoches very fine sandy loam, 100 g.-----	0-8	.90	0 .04 .08 .16	5 48 3 76 2 13 .29	5 73 3 65 2 30 .43	5 17 3 46 2 11 -----	5 46 3 62 2 18 .36	.065	.054

TABLE 2.—*Effects of increasing quantities of soil in soil-sand mixtures on the toxicity of calcium arsenate—Continued*

## EXPERIMENT 8 (MAY 20 TO JUNE 19)

Laboratory no	Kind of soil mixed with quartz sand and quantity in terms of colloid content per pot	Depth	P <sub>2</sub> O <sub>5</sub> applied per pot	Cal-cium arse-nate added per pot, ex-pressed as As <sub>2</sub> O <sub>5</sub>	Oven-dry yield of plants per pot				As <sub>2</sub> O <sub>5</sub> re-quired to reduce yield one-half	As <sub>2</sub> O <sub>5</sub> reduc-ing yield one-half minus the blank of 0.011 g for quartz sand alone
					Series A	Series B	Series C	Average		
		Inches	Gram	Gram	Grams	Grams	Grams	Grams	Gram	Gram
B171	Marshall silt loam, 25 g.	0-13	0.15	0	5.03	4.24	4.39	4.55	0.013	0.002
				.01	3.17	2.41	1.99	2.52		
				.02	1.09	1.64	1.64	1.46		
				.05	1.18	.25	.22	.22		
B171	Marshall silt loam, 50 g.	0-13	.15	0	4.99	4.28	4.17	4.48	.013	.002
				.01	3.56	2.07	2.05	2.56		
				.02	.88	1.85	1.28	1.34		
				.05	.29	.16	.40	.28		
B171	.....do.....	0-13	.225	0	4.69	4.47	3.91	4.36	-----	-----
				.02	.94	1.84	1.42	1.40		
				0	4.59	3.99	4.20	4.26		
				.02	2.69	2.18	3.16	2.68		
B171	Marshall silt loam, 150 g.	0-13	.15	0	1.53	1.47	1.75	1.58	.029	.018
				.04	.56	.37	.66	.53		
				.08	4.38	4.26	4.32	4.32		
				0	1.42	1.47	1.35	1.41		

## EXPERIMENT 9 (AUG. 8 TO SEPT. 2)

6679	Kirvin fine sandy loam, 25 g.	12-24	0.30	0	3.85	4.02	4.35	4.07	0.041	0.030
				.02	3.25	2.91	3.41	3.19		
				.04	2.03	2.12	2.02	2.06		
				.08	.44	.46	.39	.43		
6679	Kirvin fine sandy loam, 50 g.	12-24	.50	0	4.01	3.76	3.96	3.91	.049	.038
				.04	2.25	2.20	2.12	2.19		
				.08	1.11	1.04	1.36	1.17		
				.16	.25	.20	.19	.21		
6679	Kirvin fine sandy loam, 100 g.	12-24	.80	0	3.39	3.37	3.51	3.42	.081	.070
				.06	1.93	2.51	2.19	2.14		
				.12	.92	.96	1.02	.97		
				.24	.19	.23	.18	.20		

## EXPERIMENT 13 (DEC. 9 TO JAN. 20)

6719	Vernon fine sandy loam, 25 g.	3-10	0.35	0	1.96	1.57	1.75	1.76	0.024	0.013
				.02	1.14	.83	.86	.94		
				.04	.62	.51	.64	.59		
				.08	.26	.30	.24	.27		
6719	Vernon fine sandy loam, 50 g.	3-10	.50	0	1.96	1.86	1.88	1.90	.040	.029
				.03	1.11	1.03	1.24	1.13		
				.06	.69	.59	.71	.66		
				.12	.16	.24	.21	.20		
6719	Vernon fine sandy loam, 100 g.	3-10	.85	0	1.99	1.97	1.84	1.93	.055	.044
				.04	1.11	1.04	1.22	1.12		
				.08	.63	.81	.78	.74		
				.16	.24	.21	.25	.23		

Calcium arsenate is obviously highly toxic to millet in sand and in these soil-sand mixtures. The 0.011 g of As<sub>2</sub>O<sub>5</sub> application per pot, which reduces the yield one-half in pure quartz sand, is equivalent to about two parts As<sub>2</sub>O<sub>5</sub> per million of sand, or only 4 pounds per acre 6 inches.

The seat of arsenic injury is evidently chiefly in the leaves, as the arsenic-poisoned plants in all cases showed a root development that

seemed normal for the size of the plants. When sufficient calcium arsenate was applied to reduce growth markedly the leaves yellowed, became somewhat striated, and tended to wither; but no particular feature could be singled out as especially distinctive of arsenic injury. Millet, however, does not show nutritional disturbances in such characteristic ways as do tobacco and other leafy plants.

Although the critical quantities of arsenic shown in tables 1 and 2 are in all cases small, there is a rather wide range in the effects of the different soil colloids. The Barnes, Stockton, and Houston colloids in 1-percent mixtures have almost no effect in reducing the toxicity of calcium arsenate, the critical values for these colloids being only 0 to 0.007 g greater than the blank. On the other hand, in the Hagerstown, Columbiana, and Nipe soil-sand mixtures the critical arsenic values are 0.068 to 0.092 g greater than the blank. The extreme range in critical quantities of the samples tested is 0.092 g.

The comparative effects of the surface-soil and subsoil colloids on calcium arsenate may be judged from the data given in tables 1 and 2. In 11 instances the subsoil, or B horizon, was tested as well as the topsoil, or A horizon. Three subsoil colloids, those of the Marshall, Clarksville, and Norfolk soils, were from two to four times as effective as the corresponding topsoil colloids in reducing arsenic toxicity; but in the case of eight soils, the Houston, Wabash, Vernon, Kirvin, Ontario, Chester, Manor, and Caribou, there was no appreciable difference between the effects of surface-soil and subsoil colloids. The B<sub>3</sub> horizons of the Houston and Vernon soils were tested, and these had the same effects as the A and B<sub>1</sub> horizons. This general similarity in the effects of the colloids in different horizons on arsenic is unlike the results obtained with phosphate. In the phosphate study 11 of the 12 subsoil colloids were found to depress the efficiency of the phosphate more than the corresponding topsoil colloid.

Differences in the critical arsenic values cannot at this point be attributed solely to the effects of the different colloids, since the quantity of phosphate applied to the different soil-sand mixtures was varied according to the capacity of the colloid for rendering phosphate unavailable. Early in this work it seemed that the arsenic values might be influenced by the quantity of phosphate applied or by the time of application. If the phosphate were allowed to react with the colloid before the arsenate, or if an excess of phosphate were applied, it seemed possible that the capacity of the colloid for fixing anions might be satisfied by phosphate and the fixation of arsenate might be diminished thereby. Two preliminary tests, however, indicated that it made little difference whether the phosphate and arsenate were applied at the same time or whether one was allowed to react with the soil before the other by alternate wetting and drying. Also, an excess of phosphate apparently had no effect on the toxicity of calcium arsenate, for in three tests with the Nacogdoches and Marshall soils (shown in table 2) an increase in the normal phosphate application by one-half did not significantly alter the yields of the check plants or of plants receiving arsenic.

This failure of the phosphate fertilization to affect the toxicity of calcium arsenate is in accord with results obtained by Antoniani and Fonio (4), who found that phosphate ions adsorbed by soils were largely displaced by arsenate ions. Activated charcoal also adsorbs

arsenate ions more strongly than phosphate ions, according to Schilow and Lepin (15).

Since the toxicity of calcium arsenate is apparently not affected by variation in the phosphate fertilization, differences in the critical arsenic values may be attributed to differences in the effect of the soil colloids. Obviously, therefore, one of the more important factors in the resistance of soils to arsenic injury is the character of the colloidal material present. Another major factor is, of course, the quantity of colloidal material in the soil.

#### EFFECTS OF INCREASING QUANTITIES OF SOIL COLLOIDS ON THE TOXICITY OF CALCIUM ARSENATE

Four experiments were conducted to determine how the toxicity of calcium arsenate varies with increasing quantities of colloid in the sand-soil mixture. The results of these experiments are shown in

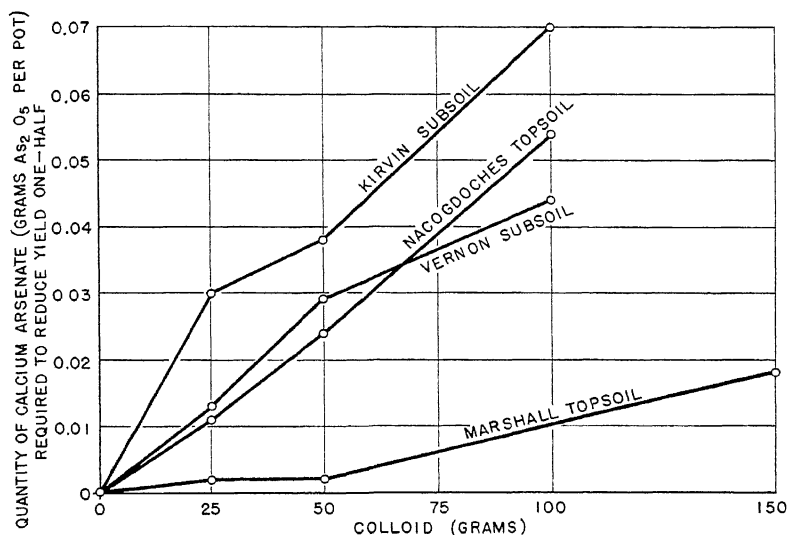


FIGURE 2.—Quantity of calcium arsenate required to reduce yield one-half, as affected by quantity of colloid.

table 2 and figure 2. There are some irregularities in the curves, but in general they approximate straight lines. Deviations are of about the magnitude of the experimental error; hence it appears that the effect of the soil colloidal material in reducing arsenic toxicity increases in approximately direct proportion to the quantity of colloid present. The experimental results upon which this conclusion is based were obtained with soil and sand mixtures containing from one-half to 3 percent of colloidal material. Although the structure of these mixtures varied somewhat, they were all open as compared with structures that might be found in highly colloidal soils. Possibly with mixtures containing from 10 to 30 percent of colloidal material, which would have a closer structure, the proportionality between percentage of colloid and effect on arsenic toxicity would not hold so well.

According to the preceding results, the arsenic resistance of a whole soil would be approximately equal to the percentage of colloid times a figure representing the resistance of the colloid. It is of interest to calculate on this basis the arsenic resistance that whole soils might have. The resulting figures are probably not accurate measures of the arsenic tolerance under field conditions of the soils used in these tests, but they should give a rough idea of the variations in tolerance that might be found in widely different soils.

The figures of tables 1 and 2, representing the quantities of  $\text{As}_2\text{O}_5$  required to reduce yields one-half, may be used for the specific resistance of 1 percent of colloid. The percentages of colloid in the different soils are given in other publications. The weight of an acre of soil to a depth of 6 inches is taken as 2,000,000 pounds. The pure quartz sand, containing no colloid, would, as already stated, show marked injury with only 4 pounds of  $\text{As}_2\text{O}_5$  per acre. The Nipe soil, on the other hand, with 60 percent or more colloid, having an exceedingly high resistance, would require 2,112 pounds of  $\text{As}_2\text{O}_5$  per acre to produce the same degree of injury. An average soil with 20 percent of colloid having an average arsenic value (the mean for colloids tested in this study) would show marked injury from an application of 192 pounds of  $\text{As}_2\text{O}_5$  per acre.

These figures should be taken as indicating only approximately the order of variation that might be found in soils of the most widely different types. Figures for the arsenic resistances of the whole soils used in this work have not been tabulated because further work would be needed to establish their reliability for field conditions. Probably some factor would be needed to convert values obtained in pot experiments to values applicable to field crops. In pot cultures root growth is altered more or less, and under field conditions there are the modifying effects of leaching, biological activity, and fluctuations in soil moisture.

#### CHARACTERISTICS OF SOIL COLLOIDS AFFECTING THE TOXICITY OF CALCIUM ARSENATE

Since the soil colloids tested in this work covered a wide range in properties, some evidence can be obtained from the data as to what characteristics of the colloids are responsible for the effects on calcium arsenate.

Apparently the effect of the colloid on calcium arsenate was not connected with acidity in any important degree. The different colloids had acidities ranging from pH 4.2 to pH 8.2, but the arsenic values showed no correspondence to these variations. For example, two soil-sand mixtures of pH 8.1 and pH 4.7, respectively, had approximately the same effect on calcium arsenate, and two other soils, both of pH 4.7, had arsenic values of 0.015 and 0.042. In view of these results acidity could be only a minor factor in arsenic toxicity.

That the effects of the colloids on calcium arsenate are connected with the chemical composition of the materials becomes evident on correlating the arsenic values with features of the ultimate analysis. Table 3 shows that the molecular ratio of silica to sesquioxides, the molecular ratio of iron to silica plus alumina, and the simple percentage of iron all have a significant correspondence with the arsenic values. Other figures of the ultimate analysis of the colloid that were

tried showed considerably less correspondence with arsenic values than the three given in table 3, and a closer correlation with other ratios of constituents is not to be expected after inspecting the figures shown in this table. The marked exceptions to the three significant correlations are the colloids of the Norfolk, Davidson, and Caribou surface soils (nos. 183, 4439, and 10541). It is hard to conceive of a combination of the analytical figures which would bring these colloids into agreement with the arsenic values without throwing the other colloids into marked disagreement.

TABLE 3.—Correspondence between arsenic values of soils and the chemical compositions of the soil colloids

Laboratory no.	Kind of soil	Depth	Arsenic value	Molecular ratio of $\text{SiO}_2$ to $\text{Al}_2\text{O}_3 + \text{Fe}_2\text{O}_3$ of colloidal material	Percentage of iron ( $\text{Fe}_2\text{O}_3$ ) of colloidal material	Molecular ratio of $\text{Fe}_2\text{O}_3$ to $\text{SiO}_2 + \text{Al}_2\text{O}_3$ of colloidal material
		<i>Inches</i>				
10305	Barnes loam.....	0-9	0.000	3.30	9.97	0.065
6097	Houston black clay.....	14-20	.001	3.24	5.92	.043
305	Stockton clay adobe.....	0-38	.001	2.87	10.57	.063
6096	Houston black clay.....	0-3	.003	3.26	5.92	.041
6098	do.....	24-36	.003	3.25	5.95	.042
183	Norfolk fine sandy loam.....	0-8	.006	1.67	11.25	.073
B3632	Houston black clay.....	1-15	.007	3.26	5.92	.041
B171	Marshall silt loam.....	0-13	.007	2.91	8.94	.057
10082	Carrington loam.....	0-3	.007	2.75	7.75	.049
10341	Miami silt loam.....	0-1 1/2	.007	2.50	7.40	.072
190	Wabash silt loam.....	15-36	.013	3.33	9.57	.057
6718	Vernon fine sandy loam.....	0-3	.014	2.65	9.87	.068
189	Wabash silt loam.....	0-15	.015	3.16	9.75	.058
4439	Davidson loam.....	0-9	.016	1.50	12.40	.088
512	Sharkey clay.....	0-4	.020	3.23	8.79	.051
B172	Marshall silt loam.....	13-24	.020	2.88	10.03	.062
298	Manor loam.....	7-20	.020	1.81	10.29	.065
297	do.....	0-7	.020	1.74	10.33	.068
9415	Cecil clay loam.....	0-6	.020	1.27	14.57	.077
195	Clarksville silt loam.....	0-10	.021	2.18	10.05	.065
6720	Vernon fine sandy loam.....	10-27	.022	2.34	10.53	.065
6719	do.....	3-10	.023	2.44	10.70	.068
184	Norfolk fine sandy loam.....	12-36	.023	1.84	11.28	.071
5028	Nacogdoches very fine sandy loam.....	0-8	.029	1.08	28.05	.282
299	Chester loam.....	0-8	.035	1.77	15.93	.115
6679	Kirvin fine sandy loam.....	12-24	.038	1.74	14.87	.094
181	Ontario loam.....	0-12	.040	2.13	10.84	.079
10543	Caribou loam.....	4-6	.040	1.41	17.63	.159
300	Chester loam.....	8-32	.042	1.79	11.84	.073
6678	Kirvin fine sandy loam.....	0-12	.045	2.02	14.74	.096
182	Ontario loam.....	12-23	.046	2.08	15.27	.100
196	Clarksville silt loam.....	10-36	.047	2.10	11.51	.072
10541	Caribou loam.....	34-2	.053	2.95	4.83	.032
391	Hagerstown loam.....	0-8	.068	1.91	9.45	.062
9804	Columbiana clay.....	0-10	.082	.81	15.35	.133
5891	Nipe clay.....	0-12	.092	.31	62.51	1.185

Coefficient of correlation of arsenic values with molecular ratio,  $\frac{\text{SiO}_2}{\text{Al}_2\text{O}_3 + \text{Fe}_2\text{O}_3}$

-0.68 ± 0.06

Coefficient of correlation of arsenic values with percentage of iron ( $\text{Fe}_2\text{O}_3$ )

+ .59 ± .07

Coefficient of correlation of arsenic values with molecular ratio,  $\frac{\text{Fe}_2\text{O}_3}{\text{SiO}_2 + \text{Al}_2\text{O}_3}$

+ .55 ± .08

The correlation coefficients given in table 3, together with their probable errors, show that within the limits of probability the two molecular ratios involving iron and the simple percentage of iron all agree about equally well with the arsenic values. Thus it seems that

iron is the chief constituent of the colloid affecting the toxicity of calcium arsenate. This conclusion is supported also by previous work with artificial inorganic gels. Biltz (5) found that silica gel had practically no effect on arsenic acid and that an alumina gel adsorbs much less than an iron gel. The activity of an iron gel, however, diminishes with age, according to Biltz (5) and to Lockemann and Lucius (12).

If iron is the only constituent of the colloid having an appreciable effect on calcium arsenate it might seem that the correlation between arsenic values and percentage of iron or ratios involving iron should be higher than 0.55 to 0.68. However, if the reactivity of the iron in the different colloids varies and does not vary in proportion to the total quantity of iron, a closer correlation could hardly be expected. No data are available on the reactivity of the iron in different colloids, but presumably it is more or less variable. One would expect the iron to be especially reactive in Podzol soils, for instance, since the transfer of iron from the surface stratum to lower layers is especially active in this group of soils. The Caribou, the only true Podzol tested, had an especially high arsenic value for its iron content.

Without attempting to follow the mechanism of the reaction in detail, it may be considered that the soil colloid reduces the toxicity of calcium arsenate by forming an insoluble ferric arsenate with any arsenate ions going into solution. This may be the essence of the matter; but the process is presumably very complicated in detail, several phases being involved—the plant root, the soil colloid, water, and particles of calcium arsenate which are insoluble. Correspondingly, the so-called “arsenic values” may be regarded as representing the quantities of arsenic combined with the colloids in a form so insoluble as to be unavailable to the plant; whereas the actual state of affairs may be somewhat different.

If the alumina of the colloid has no effect on the toxicity of calcium arsenate, it would seem that both correlations of arsenic values with percentages of iron and with ratios of iron to silica plus alumina should be higher than the correlation with ratios of silica to sesquioxides, since this last ratio includes alumina in the same term with iron. But, as a matter of fact, the silica-sesquioxide correlation is at least equal to, if not slightly closer than, the other two correlations. This may be due to the fact that not only the quantity of iron but apparently also the condition of the iron varies approximately with the silica-sesquioxide ratio. At all events, after organic matter is destroyed by hydrogen peroxide most colloids having high silica-sesquioxide ratios are gray, whereas most of those having low ratios are yellow or red. It is possible, therefore, that the silica-sesquioxide ratio evaluates roughly both quantity and reactivity of the iron.

#### EFFECTS OF SOIL COLLOIDS ON CALCIUM ARSENATE COMPARED WITH EFFECTS ON SUPERPHOSPHATE

Some of the ideas that have been offered regarding the behavior of soil colloids toward arsenate and phosphate are supported by the comparative quantities of arsenate and phosphate rendered unavailable. These ideas, to recapitulate briefly, are that reaction of the colloid with arsenate is dependent only on the iron, whereas reaction with phosphate is dependent on the phosphate content of the colloid



and on both iron and alumina. A previous publication (10) gives the quantities of phosphate fixed by a number of soil colloids, some of which were used in this work.

That the phosphate content of the colloid diminishes fixation of phosphate but does not affect fixation of arsenate is in accord with the comparative effects of topsoil and subsoil colloids on the two substances. It was found in the superphosphate study that nearly all topsoil colloids fix less added phosphate than the corresponding subsoil colloids. This was explained on the basis that the topsoil colloid in nearly all cases is higher in phosphorus than the subsoil colloid and is therefore more nearly saturated with phosphate. Granting that this idea is correct, it would follow that the phosphate content of the colloid has little or no effect on arsenate adsorption, since, as previously mentioned, the topsoil and subsoil colloids in most cases fix equal quantities of arsenate.

The conclusion that arsenate fixation is dependent on the iron of the colloid, while phosphate fixation is dependent on both alumina and iron is supported by certain correlations. In the first place, there is a significant but not close correlation between the quantities of arsenate and of phosphate fixed by a number of soil colloids. The correlation coefficient between arsenate and phosphate values is 0.54 for 13 surface-soil colloids and 0.69 for 6 subsoil colloids. Coefficients of this magnitude are about what one would expect if one series of values were dependent on iron alone and the other series on both iron and alumina. The phosphate content of the colloid as a factor affecting fixation of phosphate was largely eliminated by correlating the phosphate values of surface-soil colloids and subsoil colloids separately with the arsenate values.

Further correlations furnish more direct evidence that the arsenate values are dependent on iron alone and that phosphate values are dependent on both iron and alumina. The arsenate values show a fair correlation with iron alone or with iron plus alumina but practically no correspondence with alumina alone. The correlation coefficients are 0.59 for iron, 0.68 for iron plus alumina, and only 0.20 for alumina. Phosphate values, on the other hand, show a fair correspondence with both iron and alumina alone and a fairly close correspondence with iron plus alumina. Correlation coefficients obtained on comparing phosphate values with the chemical constituents of topsoil colloids and of subsoil colloids, separately, were respectively: 0.42 and 0.50 with iron alone, 0.55 and 0.69 with alumina alone, and 0.71 and 0.79 with iron plus alumina.

#### SUMMARY

This investigation deals with the comparative effects of different soil colloids on the toxicity of calcium arsenate to millet.

Effects of the colloids are determined from comparative growths made by millet (*Setaria italica*) in pot cultures when increasing quantities of calcium arsenate are added to pure quartz sand and to soil-sand mixtures. The soil-sand mixtures contain sufficient soil to supply 1 percent of colloidal soil material. The quantity of calcium arsenate (expressed as  $\text{As}_2\text{O}_5$ ) required to reduce the yield one-half in the soil-sand mixture minus the quantity required to reduce the yield

one-half in pure quartz sand is taken as the measure of the effect of the colloid and is called the "arsenic value" of the colloid.

The arsenic values of 36 soil colloids tested show a wide range of variation; namely, from 0.000 to 0.092 g of  $\text{As}_2\text{O}_5$  per pot. In other words, in some soil-sand mixtures the quantity of calcium arsenate reducing the yield one-half is the same as in pure quartz sand (0.011 g of  $\text{As}_2\text{O}_5$  per pot); whereas in another soil-sand mixture the quantity of arsenate reducing the yield one-half is 0.092 g of  $\text{As}_2\text{O}_5$  more than the 0.011 g of  $\text{As}_2\text{O}_5$  required in quartz sand.

The effect of the soil colloid in reducing toxicity of calcium arsenate increases in approximately direct proportion to the quantity of colloid, at least up to a few percent of colloid. The resistance of a soil to calcium arsenate injury, therefore, depends on the quantity of colloid present and on the specific resistance of the colloid.

On the basis of these pot experiments it appears that whole soils could show resistances to calcium arsenate injury as variable as the following: Soils that are made up of practically pure quartz sand, 4 pounds of  $\text{As}_2\text{O}_5$  per acre; soils with 20 percent of colloid of average arsenic resistance, 192 pounds of  $\text{As}_2\text{O}_5$ ; and soils containing 60 percent of colloid of exceptionally high specific resistance, 2,112 pounds of  $\text{As}_2\text{O}_5$ . These figures may have no practical significance except as indicating possible degrees of variation for certain plants.

The effects of the colloid on calcium arsenate appears to be uninfluenced by the phosphate fertilization, by the acidity of the colloid, and by the presence of calcium carbonate, but it does appear to be influenced by the chemical composition of the colloid. The arsenic values of the colloids correspond roughly with the silica-sesquioxide ratios of the colloids, with the simple percentage of iron, and with the ratio of iron to silica plus alumina: the coefficients of correlation with these features of the chemical composition are, respectively,  $-0.68 \pm 0.06$ ,  $+0.59 \pm 0.07$ , and  $+0.55 \pm 0.08$ . It is concluded that the effect of the colloid is dependent on the quantity and reactivity of the iron present.

A comparison of the arsenic values with figures previously obtained for phosphate rendered unavailable by soil colloids tends to support the conclusion that the effect of the colloid on calcium arsenate is dependent only on the iron, whereas the effect on superphosphate is dependent on the phosphate, iron, and alumina contents of the colloid.

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# THE EFFECTIVENESS OF VARIOUS ARSENICALS IN DESTROYING LARVAE OF THE JAPANESE BEETLE IN SASSAFRAS SANDY LOAM <sup>1</sup>

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## INTRODUCTION

In a previous contribution from the Japanese beetle laboratory (3)<sup>3</sup> it was shown that larvae of the Japanese beetle (*Popillia japonica* Newm.) were killed by the introduction of certain arsenicals into the soil. Subsequent investigations have been carried on to determine the effectiveness of different arsenicals for this purpose, when freshly applied and after having been in the soil for several years. In the study of the insecticidal action in Sassafras sandy loam during the period 1929 to 1935, which is reported in this paper, over 18,000 tests were made, involving more than 90,000 larvae.

Larvae of the Japanese beetle may be killed upon ingesting arsenic while burrowing through poisoned soil or feeding on roots growing in this soil. When they have a lethal quantity, the larvae cease feeding and burrowing, discharge the contents of the alimentary tract, become flabby, and eventually die. The period of time required for the arsenical to kill depends upon its nature and concentration and upon the development, activity, and susceptibility of the larvae. The nature and concentration of the material applied to soil can be controlled, the larvae of a definite stage of development can be selected, and the activity of the insects can be controlled to a large extent by regulating the food, temperature, and moisture in the soil, but it is impossible to obtain, in different fields, or from the same field at different periods of the year, groups of larvae that have the same susceptibility to arsenic. In any study of the effectiveness of a material as a stomach-poison insecticide for the larvae of the Japanese beetle, it is necessary to conduct the tests under conditions in which the larvae are active, and to compare the killing power of the material with that of a standard stomach-poison insecticide, such as acid lead arsenate, under the same conditions on larvae from the same source.

## ARSENICALS USED

The arsenic trioxide used in this study was not analyzed but was guaranteed by the manufacturer to be 98 to 100 percent pure. The other arsenicals had the composition<sup>4</sup> shown in table 1. The names used to designate the various samples are those employed by the

<sup>1</sup> Received for publication Oct. 23, 1935; issued April, 1936.

<sup>2</sup> The writers are indebted to F. M. Wadley, of the Bureau of Entomology and Plant Quarantine, for assistance in the statistical treatment of the data.

<sup>3</sup> Reference is made by number (italic) to Literature Cited, p. 503.

<sup>4</sup> Analyses of these materials were made by the Insecticide Division when it was a division of the Bureau of Chemistry and Soils.

manufacturers and do not imply that definite, pure salts were available. In particular, the two calcium arsenates are to be regarded as containing considerable of the compounds named, but in admixture with such quantities of excess lime and calcium carbonate as to make their gross analyses practically identical.

#### CONDITIONS FOR TESTING STOMACH-POISON INSECTICIDES ON LARVAE

It has been observed that when the temperature of the soil is below 50° F. the larvae of the Japanese beetle are largely inactive and are affected by stomach poisons only to a limited extent. As the temperature of the soil is raised to 85°, the activity of the larvae increases and the period of time required for an insecticide to become effective is progressively decreased. At a temperature of 80° to 85° a stomach poison may be effective within 2 weeks, whereas at 60° from 6 to 8 weeks may be required. Temperatures above 85° appear to retard the activity of the larvae. Activity is also impaired in soils that are excessively dry or wet.

TABLE 1.—Percentage composition of the arsenates used in this study

Arsenate	Moisture	Total arsenic oxide	Total arsenious oxide	Water- soluble arsenic oxide	Total base
Aluminum.....	7.28	33.71	0.69	0.72	39.82 as $Al_2O_3$ .
Barium.....	.56	26.69	.10	2.38	65.55 as BaO
Dicalcium.....	1.05	40.15	( <sup>1</sup> )	7.00	40.74 as CaO
Tricalcium.....	1.31	40.64	.17	.20	40.57 as CaO
Ferric.....	2.28	47.64	.16	1.19	33.86 as $Fe_2O_3$ .
Acid lead.....	.14	32.41	.28	.41	63.13 as PbO
Basic lead.....	.32	22.09	.15	.17	72.59 as PbO
Magnesium.....	3.79	32.84	.05	.61	39.58 as MgO
Manganese.....	1.40	42.97	.00	1.02	48.32 as MnO
Zinc.....	.91	33.07	3.60	.34	57.94 as ZnO

<sup>1</sup> Trace

It was decided to test the effectiveness of stomach poisons at a constant temperature of 80° to 85° F. in Sassafra sandy loam about half saturated with water and to supply an abundance of food by sowing grain in the soil. These tests were conducted in large chambers maintained at a relative humidity of 96 to 100 percent at the desired temperature to prevent excessive loss of moisture from the soil, and lighted artificially to promote the growth of the grain. A description of these chambers has been published (2, p. 124).

#### PROCEDURE FOR CONDUCTING A TEST

In every test commercial acid lead arsenate, which was used as a standard insecticide because several years' experience with the material in the field had demonstrated its effectiveness in killing Japanese beetle larvae, was applied to the soil at the rates of 250, 500, 1,000, 1,500, and 2,000 pounds per acre within a few hours before introduction of the larvae. In mixing, 0.735 cubic foot of soil, sufficient to fill twenty 5-inch earthen pots, was first spread in a thin layer on a smooth concrete floor, and then a weighed quantity of the lead

arsenate was scattered uniformly over the soil and mixed with it by raking and shoveling until uniformly dispersed. The quantities of material added to this volume of soil and the equivalent number of pounds per acre when mixed to a depth of 3 inches are as follows:

Grams per 0.735 cubic foot:	Equivalent pounds per acre to a depth of 3 inches
7.3.....	250
14.7.....	500
29.4.....	1,000
44.1.....	1,500
58.8.....	2,000

After each batch had been mixed, it was placed in twenty 5-inch pots, the drainage holes of which were covered with 16-mesh wire to prevent the subsequent escape of larvae. An equal number of pots were filled with untreated soil for the determination of the natural mortality of the insect. Other materials were then applied in the same manner, or pots of soil that had been treated previously and exposed to weathering in the field were brought in and the soil was carefully examined to remove any larvae that might be present as a result of natural infestation. Grain was sown in each pot.

The pots were plunged to a depth of 2 inches in moist peat in the constant-temperature chamber. After 48 to 72 hours, when the soil was at the proper temperature and the grain had sprouted, five active third-instar larvae were placed in each pot. The most satisfactory procedure for infesting the pots is to make five holes 2 inches deep with a small dibble, place one larva in each hole, and fill the holes with soil. Third-instar larvae were selected because larvae in this stage are most resistant to insecticides; in addition, it is easier to handle larvae of this age without injuring them, and such larvae are more readily seen in soil during the examination than are those of other instars. It was not feasible, because of cannibalism, to place more than five larvae in a 5-inch pot of soil.

Seven days after the larvae had been introduced, the soil was removed from each pot and the numbers of dead and living were recorded. The soil and the living larvae were then replaced in the pot, the dead being discarded. After being left undisturbed for a second 7-day period, the potted soil was reexamined and the condition of the larvae recorded a second time.

At the end of the first 7-day period usually half of the larvae were surviving in soil treated with the standard insecticide, commercial acid lead arsenate, at rates of 1,000 to 1,500 pounds per acre, and at the end of the second period this condition existed with concentrations of 500 to 1,000 pounds. When a material was considerably more effective than the standard insecticide, usually half of the larvae were alive at concentrations between 250 and 1,000 pounds per acre at the end of the first period and few were alive in any concentration at the end of the second period. When a material was considerably less effective than the standard, the point of median survival was found between concentrations of 1,000 and 2,000 pounds at the end of the second period. The materials that were considerably more effective than the standard were therefore compared with the standard at the end of the first 7-day period, and the other materials were compared at the end of the second period.

## EFFECTIVENESS OF VARIOUS ARSENICALS

It has been shown (6, 7, 8) that the closest agreement in tests with insecticides occurs when the 50-percent death point is selected as the criterion. The method used in determining the median points of survival and the coefficient of effectiveness of the different treatments is illustrated in the determination of the effectiveness of aluminum arsenate that had been freshly applied to the soil. The average number of larvae surviving in each concentration of aluminum arsenate and in each concentration of the standard insecticide tested at the same time, their respective checks, and their standard errors <sup>5</sup> are given in table 2.

TABLE 2.—Average number of larvae surviving from an original of 5 larvae per pot in various concentrations of aluminum arsenate as compared with their survival in the standard insecticide

Quantity of material (pounds per acre)	Average number of larvae surviving in—	
	Standard insecticide	Aluminum arsenate
0.....	3.73±0.123	3.73±0.123
500.....	2.91±.125	2.16±.173
1,000.....	1.55±.172	1.33±.176
1,500.....	.77±.105	.65±.127
2,000.....	.47±.113	.61±.119

Since an average of 1.27 larvae were dead in the pots of untreated soil in this series, it is considered that in each concentration of these materials this number had died from causes other than poisoning, such as injury or disease. One-half the survival in the untreated soil was therefore selected as the median survival point, or where 50 percent were killed by poisoning. The median survival in this case was found to be  $1.86 \pm 0.161$  larvae. <sup>6</sup>

An examination of the data for the standard insecticide shows that the concentration where 50 percent were killed by poisoning is between 500 and 1,000 pounds per acre. If  $A$  is the concentration giving a survival,  $C$ , just above the median survival,  $M$ , and  $B$  is the concentration giving a survival,  $D$ , just below the median survival, the median effective concentration of the standard,  $M.C.$ , can be estimated by substituting the required values in the equation <sup>7</sup>

<sup>5</sup> It should be noted that the standard error, and not the probable error, is used throughout this paper. The standard error of the mean was calculated according to the equation  $\sigma_m = \frac{\sigma_x}{\sqrt{N-1}}$ , where  $\sigma_x$  is the standard deviation and  $N$  is the number of pots.

<sup>6</sup> The standard error of the median survival in the soil containing an insecticide cannot be determined from the error of the survival in untreated soil. It was therefore estimated according to the procedure outlined by Trevan (8). The percentage errors of the survivals above and below the median were plotted and the percentage error of the median obtained by interpolation. The error of the median was then calculated from the value of the median.

<sup>7</sup> This equation for the median effective concentration was derived in the following manner: The median concentration can be expressed by the equation

$$M.C. = A + (B - A) \left[ \frac{C \pm c - M \pm m}{C \pm c - D \pm d} \right]$$

As the samples are from different concentrations, however, the standard error of the difference is equal to the square root of the sum of the squares of the errors of the proportions (4, p. 214), and this equation becomes

$$M.C. = A + (B - A) \left[ \frac{C - M \pm \sqrt{c^2 + m^2}}{C - D \pm \sqrt{c^2 + d^2}} \right]$$

When these values are substituted in the equation to determine the error of a quotient (communication from Sewall Wright to B. A. Porter, Bureau of Entomology and Plant Quarantine), the equation becomes

$$M.C. = A + (B - A) \left[ \frac{C - M}{C - D} \pm \frac{C - M}{C - D} \sqrt{\frac{c^2 + m^2}{(C - M)^2} + \frac{c^2 + d^2}{(C - D)^2}} \right]$$



$$M.C._s \pm e = A + (B - A) \left[ \frac{C - M}{C - D} \pm \frac{C - M}{C - D} \sqrt{\frac{c^2 + m^2}{(C - M)^2} + \frac{c^2 + d^2}{(C - D)^2}} \right]$$

$A$  in this case equals 500,  $B$  equals 1,000,  $C$  equals 2.91,  $c^2$  equals 0.125<sup>2</sup>,  $M$  equals 1.86,  $m^2$  equals 0.161<sup>2</sup>,  $D$  equals 1.55, and  $d^2$  equals 0.172<sup>2</sup>. The median effective concentration of the standard was found to be 886 ± 96 pounds, and in like manner the median effective concentration of freshly applied aluminum arsenate was found to be 681 ± 156 pounds.

The coefficient of effectiveness of the aluminum arsenate was then determined by the equation

$$\text{Coefficient of effectiveness} = \frac{M.C._s \pm e}{M.C._t \pm e_1} = \frac{M.C._s}{M.C._t} \pm \frac{M.C._s}{M.C._t} \sqrt{\frac{e^2}{(M.C._s)^2} + \frac{e_1^2}{(M.C._t)^2}}$$

where  $M.C._s \pm e$  is the median effective concentration of the standard insecticide and its error and  $M.C._t \pm e$  is the median effective concentration of the aluminum arsenate and its error. The coefficient of effectiveness of freshly applied aluminum arsenate was found to be 1.30 ± 0.329, indicating that when freshly applied this material is 1.30 ± 0.329 times as effective as freshly applied acid lead arsenate in killing the larvae in the soil.

The coefficients of effectiveness of the various arsenicals in killing larvae were determined immediately after application and at intervals up to 60 months after application. The coefficients are given in table 3.

TABLE 3.—*Coefficients of effectiveness of various arsenates<sup>1</sup> and arsenic trioxide against larvae of the Japanese beetle after the arsenicals had been in the soil for different periods of time*

Months in the soil	Coefficient of effectiveness				
	Aluminum arsenate	Barium arsenate	Dicalcium arsenate	Tricalcium arsenate	Ferric arsenate
0.....	1.30±0.329	1.10±0.216	1.80±0.339	1.46±0.310	1.04±0.140
6.....	.94±.284	1.09±.233	2.44±.599	1.21±.196	.78±.149
12.....	1.09±.248	.75±.134	1.24±.243	1.13±.216	.72±.142
18.....	1.00±.279	.70±.326	1.45±.359	1.28±.280	.66±.132
24.....	.87±.477	.96±.214	1.27±.259	.80±.165	.42±.165
30.....	.78±.133	.67±.121	.96±.216		.42±.194
31.....				1.02±.204	
43.....	.98±.125	.68±.112	.77±.098	.94±.154	
44.....					35±.254
48.....	0	0	0	0	0
60.....	0	0	0	0	0

Months in the soil	Coefficient of effectiveness				
	Acid lead arsenate	Magnesium arsenate	Manganese arsenate	Zinc arsenate	Arsenic trioxide
0.....	1.00±0.148	1.89±0.438	1.97±0.449	1.16±0.181	1.85±0.344
6.....	.98±.159	1.14±.193	.88±.287	.83±.208	2.75±.547
12.....	.76±.118	1.38±.203	1.33±.277	1.00±.186	2.00±.530
18.....	.65±.100	.97±.163	1.05±.274	.92±.162	2.03±.427
24.....	.63±.110	.87±.333	.82±.132	.75±.178	2.39±.768
30.....	.53±.102				2.36±.426
31.....		.83±.105	.92±.128	.68±.090	
43.....					2.77±.431
44.....	.25±.056	.31±.061	.37±.070	.33±.073	
48.....	0	0	0	0	1.30±.261
60.....	0	0	0	0	1.34±.233

<sup>1</sup> Basic lead arsenate was tested as extensively as the other materials, but the survival was so high, even when used at the rate of 16,000 pounds per acre, that it was not possible to determine the median effective concentration. The coefficient of effectiveness was consistently zero.

## DISCUSSION OF RESULTS

When the coefficients of effectiveness of the various materials were plotted against the time that the materials had been in the soil, the functional relationship could be expressed by means of a straight line. To determine accurately the position of this line, the constants of the equation of the line were determined mathematically by the method of least squares (1, pp. 55-61). The coefficient of correlation (1, pp. 121, 128),  $\bar{r}_{xy}$ , adjusted for the number of cases, the adjusted standard error of estimate (1, pp. 121, 128),  $\bar{S}_{yx}$ , and the coefficient of determination (1, p. 120) were then calculated (table 4). The

TABLE 4.—Statistical measures showing the association between the coefficients of effectiveness of various arsenicals and the time these arsenicals remained in the soil

Arsenical	Adjusted coefficient of correlation	Coefficient of determination	Standard error of estimate <sup>1</sup>	Arsenical	Adjusted coefficient of correlation	Coefficient of determination	Standard error of estimate <sup>1</sup>
Aluminum arsenate.....	0.793	0.629	0.284	Acid lead arsenate.....	0.942	0.887	0.128
Barium arsenate.....	.855	.731	.213	Magnesium arsenate.....	.946	.895	.204
Dicalcium arsenate.....	.870	.757	.391	Manganese arsenate.....	.866	.750	.318
Tricalcium arsenate.....	.841	.707	.289	Zinc arsenate.....	.946	.895	.137
Ferric arsenate.....	.883	.780	.168	Arsenic trioxide.....	.109	.012	.321

<sup>1</sup> In terms of the coefficient of effectiveness.

regression line, the standard error of estimate, and the determined coefficients of effectiveness are presented graphically in figure 1.

For the arsenates there was a high correlation between the coefficient of effectiveness and the period of time that the materials had been in the soil, but there appeared to be little such correlation for arsenic trioxide. The coefficient of determination ( $\bar{r}_{xy}$ )<sup>2</sup> is an arbitrary mathematical measure which shows what proportion of the variance in the values of the dependent variable can be explained by the concomitant variation in the values of the independent variable. It was found that almost 90 percent of the variance in the coefficients of acid lead arsenate, magnesium arsenate, and zinc arsenate could be accounted for by the period the materials had been in the soil. More than 60 percent of the variance in the coefficients of the other arsenates could be attributed to this factor. Since this leaves only a relatively small percentage of the variance to be accounted for by all other factors, it appears that the period of time in the soil was the most important factor associated with the coefficient of effectiveness of these materials. As the coefficient of determination of arsenic trioxide was only 1.2 percent, it is evident that other factors were more important than the period of time in modifying its insecticidal action.

The standard error of estimate shows how nearly the estimated values of the coefficients agree with the determined values. As most of the determined values were scattered throughout the zone  $\pm 1$  standard error from the regression line, the standard error of estimate may be used as a measure of the consistency of the insecticidal action of a material. Acid lead arsenate appears to be the most dependable of the arsenates for killing larvae in the soil; zinc arsenate and ferric arsenate were very close to acid lead arsenate in the consistency of their insecticidal action. The other arsenicals were more variable. The variation in the results obtained with aluminum

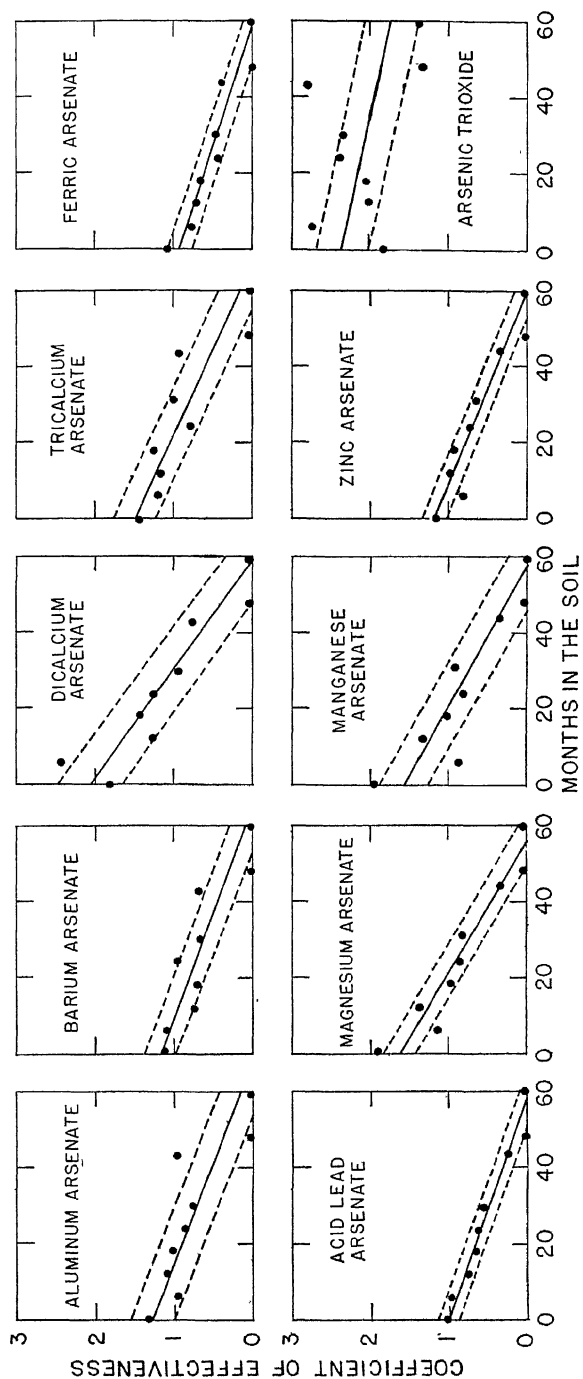


FIGURE 1.—Effectiveness of various arsenicals against larvae of the Japanese beetle after the arsenicals had been in the soil for different periods of time.

arsenate, dicalcium arsenate, tricalcium arsenate, manganese arsenate, and arsenic trioxide was so great as to raise some doubt regarding the dependability of these materials as stomach-poison insecticides in the soil.

Theoretically, when the linear correlation is high the averages derived from several observations for each period should lie along the regression line, and these averages should very nearly coincide with the estimated values. To have the average of the determined values coincide with the estimated value for each period, it would be necessary to make many tests of a stomach poison in a complex medium such as soil. In view of the long period of time and the labor involved in determining the coefficient of a stomach-poison insecticide in soil, it has not been possible, in most cases, to make more than two or three series of determinations at a given period. Therefore, some fluctuation may be expected in the average determined coefficients around the regression line. It is believed that under the circumstances the regression line, which is based on all the determined values, is probably a more accurate average and a better basis of comparison than the determined values at a given period. It was decided, therefore, to use the estimated values as a basis for comparing the insecticidal effectiveness of the different materials in the soil.

The estimated coefficients of effectiveness at 0 months measure the effectiveness of the freshly applied materials. As these coefficients are subject to variation, as are other statistical constants, the standard errors of the coefficients were determined (1, p. 253; 4, p. 77) by the equation

$$\text{Standard error of estimated coefficient of effectiveness} = \frac{\bar{S}_{yx}}{\sqrt{N}}$$

The estimated coefficients were then arranged in order of increasing difference from the coefficient of acid lead arsenate, and the differences between the coefficient of acid lead arsenate and the other arsenicals were determined as given in table 5. It is generally accepted that when a value is two or more times its standard error the value can be considered significant. It is apparent from these data that ferric arsenate is significantly less effective and the other arsenicals are significantly more effective than acid lead arsenate when freshly applied to the soil.

TABLE 5.—Significance of the difference between the estimated coefficients of effectiveness of the various arsenicals when freshly applied

Arsenical	Estimated coefficient of effectiveness	Difference between coefficient of effectiveness of acid lead arsenate and that of arsenical indicated	Ratio
Acid lead arsenate.....	1.01±0.0199		
Ferric arsenate.....	.90±.0286	0.11±0.0348	3.2:1
Barium arsenate.....	1.14±.0336	.13±.0390	3.3:1
Zinc arsenate.....	1.16±.0246	.15±.0316	4.7:1
Aluminum arsenate.....	1.28±.0449	.27±.0491	5.5:1
Tricalcium arsenate.....	1.48±.0519	.47±.0555	8.5:1
Manganese arsenate.....	1.56±.0571	.55±.0605	9.1:1
Magnesium arsenate.....	1.61±.0366	.60±.0417	14.4:1
Dicalcium arsenate.....	2.13±.0618	1.12±.0649	17.3:1
Arsenic trioxide.....	2.36±.0507	1.35±.0545	24.8:1

It was thought that there might be some correlation between the effectiveness of the various arsenicals and the percentage of total or water-soluble arsenic oxide in the materials. Apparently, however, there was no correlation between the coefficient of effectiveness and the percentage of water-soluble arsenic oxide or of arsenious oxide, although there did appear to be some relation between the coefficient of effectiveness and the total arsenic oxide. The coefficient of correlation and the coefficient of determination were calculated by the regular procedure. The coefficient of correlation was found to be 0.672 and the coefficient of determination, 0.452. As 45.2 percent of the variance in the coefficients of effectiveness could be accounted for by the total arsenic oxide content of the materials, it is apparent that the arsenic oxide content has some influence, although other properties of the materials are more important in modifying the insecticidal action.

The coefficient of regression measures the slope of the regression line; it shows the average change in the coefficient of effectiveness each month the material has been in the soil. The variation likely to be present in the coefficient of regression of the different materials was determined (1, p. 252; 8, p. 352) by the equation

$$\text{Standard error of } b_{xy} = \frac{\bar{S}_{yx}}{\sigma_x \sqrt{N}}.$$

The coefficients of regression were then arranged in the order of increasing difference from that of acid lead arsenate, and the differences were determined as given in table 6. It was found that magnesium arsenate and dicalcium arsenate decreased in effectiveness more rapidly than did acid lead arsenate, but no significant difference in the rate of change could be demonstrated with the other materials.

TABLE 6.—*Significance of the difference in the change in effectiveness of the arsenicals in the soil*

Arsenical	Coefficient of regression	Difference between coefficient of regression of acid lead arsenate and that of arsenical indicated	Ratio
Acid lead arsenate.....	0.0179±0.0022		
Barium arsenate.....	.0178±.0037	0.0001±0.0043	0.02:1
Aluminum arsenate.....	.0189±.0049	.0010±.0054	.18:1
Zinc arsenate.....	.0185±.0023	.0016±.0032	.50:1
Ferrie arsenate.....	.0159±.0029	.0020±.0036	.55:1
Tricalcium arsenate.....	.0228±.0050	.0049±.0055	.89:1
Arsenic trioxide.....	.0103±.0056	.0076±.0060	1.27:1
Manganese arsenate.....	.0277±.0055	.0098±.0059	1.66:1
Magnesium arsenate.....	.0295±.0035	.0116±.0041	2.83:1
Dicalcium arsenate.....	.0349±.0068	.0170±.0071	2.39:1

As chemical analyses showed that there was little change in the total arsenic content of the soil in the pots during a period of 60 months, the decrease in the effectiveness of the materials in this case cannot be due to removal of the material from the soil by leaching. Basic lead arsenate was found to be ineffective, even at 10 times the concentration of acid lead arsenate. It appears, therefore, that the loss

of effectiveness of these arsenicals in Sassafras sandy loam can be attributed to the slow conversion of the arsenic into a form, probably a complex basic salt, that is not toxic to the insect. In the field the decrease in effectiveness of an arsenical is influenced to some extent by leaching, and under these conditions the change in insecticidal action can be attributed to both factors.

#### SUMMARY

During the period 1929 to 1935 a study was made of the relative effectiveness of the inorganic arsenates and arsenic trioxide as stomach-poison insecticides for the destruction of the larvae of the Japanese beetle in the soil. The experiments were conducted under conditions in which temperature, moisture, and food in the soil were controlled, with larvae from the same source. More than 18,000 tests, involving 90,000 larvae, were made with aluminum arsenate, barium arsenate, dicalcium and tricalcium arsenates, ferric arsenate, acid lead arsenate, basic lead arsenate, magnesium arsenate, manganese arsenate, zinc arsenate, and arsenic trioxide.

The effectiveness of the materials in Sassafras sandy loam was determined immediately after application and at intervals up to 60 months. The median effective concentration of each material was compared with that of freshly applied acid lead arsenate, which was used as a standard insecticide and tested at the same time, and the results were expressed as the coefficient of insecticidal effectiveness of the standard.

Almost 90 percent of the variance in the coefficients of acid lead arsenate, magnesium arsenate, and zinc arsenate, and more than 60 percent of that of the other arsenates, could be accounted for by the period the materials had been in the soil, but only 1.2 percent of the variance in the coefficient of arsenic trioxide could be attributed to this factor. It is evident, therefore, that the period of time in the soil was the most important factor associated with the effectiveness of the arsenates but that other factors were more important in modifying the insecticidal action of arsenic trioxide.

Acid lead arsenate appeared to be the most dependable arsenical for killing larvae in soil, with zinc arsenate and ferric arsenate almost as consistent in their action. The variation in the results obtained with aluminum arsenate, dicalcium arsenate, tricalcium arsenate, manganese arsenate, and arsenic trioxide was so great as to raise some doubt regarding their dependability as soil insecticides.

Basic lead arsenate was found to be of no value as a soil insecticide. Ferric arsenate was significantly less effective and the other materials were significantly more effective than acid lead arsenate when freshly applied to the soil. There was no correlation between the water-soluble arsenic oxide content or the arsenious oxide content of the materials and their effectiveness. As 45.2 percent of the variance in the coefficients could be accounted for by the total arsenic oxide content of the materials, it is apparent that the arsenic oxide content has some influence, although other properties of the materials are more important in modifying the insecticidal action.

Magnesium arsenate and dicalcium arsenate decreased in effectiveness more rapidly than acid lead arsenate, but no significant difference in the rate of change could be demonstrated with the other materials.

As there was little loss in the total arsenic content of the soil under the experimental conditions during a period of 60 months, the decrease in effectiveness can be attributed to the slow conversion of the arsenic into a form, possibly a complex basic salt, that is not toxic to the insect. In the field the loss in effectiveness can properly be attributed to the removal of the arsenic by leaching and to its conversion to an ineffective form.

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# ERRORS OF ROUTINE ANALYSIS FOR PERCENTAGE OF SUCROSE AND APPARENT PURITY COEFFICIENT WITH SUGAR BEETS TAKEN FROM FIELD EXPERIMENTS<sup>1</sup>

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## INTRODUCTION

The extent and source of experimental error, for the determination of percentage of sucrose and apparent purity coefficient of sugar beets grown in experimental trials, are of considerable practical importance. Very little information is available regarding the magnitude of these errors. Generally, the percentage of sucrose and the apparent purity coefficient are determined from a composite sample of pulp rasped from 10, 20, or 30 beets included in the sample. It is important to know the rapidity with which the error decreases as the size of the sample increases. Since this error will be due to a combination of many factors entering into the analysis, it is of interest to determine also, as far as possible, the source of the major portion of the error. With such information available, it is possible to determine the lines along which improvement in technique, leading to a reduction in experimental error, may be sought.

The following study was made to ascertain the errors of routine analysis for the determination of percentage of sucrose and apparent purity coefficient in sugar-beet samples from experimental plots.

## MATERIALS AND METHODS

In 1933 seed of the Kleinwanzleben "Normal" strain of sugar beets was planted on May 7, and the beets were harvested October 4 to 6. In 1934 seed from a composite cross of several high-yielding varieties was planted on May 8, and the beets were harvested October 9 to 11.

The seed was planted in rows 20 inches apart and thinned to a single beet per 12 inches of row. The stand was satisfactory both years. All beets adjacent to obvious skips in the row were removed before harvest, and the results were based on the study of beets with normal competition on all sides.

The design of the experiment was the same in both years. The land was divided into five blocks each year. In 1933 each block was made up of 30 rows. In 1934, 4 of the 5 blocks were 40 rows wide while the fifth contained 35 rows. The plots were 66 feet long in both years. Each block was divided into 10 plots of equal size. Six lots consisting of 2 samples each of 10, 20, and 30 beets were taken at random from each plot. Care was used to sample the entire plot adequately.

<sup>1</sup> Received for publication October 12, 1935; issued April 1936. Cooperative investigations by the Division of Sugar Plant Investigations, Bureau of Plant Industry, U. S. Department of Agriculture, and the Divisions of Agronomy and Plant Genetics and Plant Pathology and Botany of the Minnesota Agricultural Experiment Station. Paper no. 1349 of the Journal Series of the Minnesota Agricultural Experiment Station.

<sup>2</sup> Resigned August 1, 1935; now associate professor of agronomy and plant genetics, University of Minnesota.

The samples were brought to the laboratory immediately. The beets were washed and then each beet was split into halves with a motor-driven circular saw. The weight of the beets, percentage of sucrose, and apparent purity coefficient were obtained for both halves of each sample (hereafter called duplicates). The duplicates were, therefore, the 10, 20, or 30 halves taken from the left or the right side of the saw.

The percentage of sucrose, by direct polarization, was determined by the Sachs-Le Docte cold-water digestion method from a composite of pulp from all the beets in each duplicate. An immersion refractometer equipped with a water jacket for temperature control was used to determine the percentage of total solids. The apparent purity coefficient (hereinafter called purity coefficient) was then calculated by dividing the percentage of sucrose by the percentage of total solids.

A crew of six washed and weighed the beets in each sample, split them into halves, rasped the halves for a composite sample of pulp, determined the saccharimeter and refractometer readings, and washed the glasses and filters after use. The persons reading the instruments had had a short period of training before starting on the regular analyses. The saccharimeter readings were made by the same person during both years; otherwise the laboratory crew was different each year. Speed in operation was stressed throughout, the analyses in this study being made at a rate of slightly over 200 sugar and purity analyses each per day of about 7½ working hours.

Two samples of 10, 20, and 30 beets each were selected from each of 10 plots within each of 5 blocks, making 100 samples of each size. When the beets included in each sample were split into halves, 200 duplicate samples resulted, thus permitting 200 readings to be made for each sample size. The entire experiment was duplicated the second year. The data were analyzed by the procedure described by Fisher<sup>3</sup> as the analysis of variance.

Analyses of variance were made, for each of the three sample sizes in each of the 2 years, for weight of beet, percentage of sucrose, and purity coefficient. Analyses of covariance were made, for each sample size, for the three variables in all combinations consisting of groups of two.

### EXPERIMENTAL RESULTS

Table 1 gives, for each sample size, the means for weight per beet (in pounds), percentage of sucrose, and purity coefficient for each year and for the average of both years.

TABLE 1.—Mean weight per beet, percentage of sucrose, and apparent purity coefficient for 10-, 20-, and 30-beet samples in each of 2 years and the average of both years

MEAN WEIGHT PER BEET (POUNDS)			
Year	10-beet sample	20-beet sample	30-beet sample
1933.....	1.5344	1.5274	1.4492
1934.....	.9330	.8714	.9235
Average.....	1.2337	1.1994	1.1864

<sup>3</sup> FISHER, R. A. STATISTICAL METHODS FOR RESEARCH WORKERS. Ed 5, rev. and enl., 319 pp., illus. Edinburgh and London. 1934

TABLE 1.—Mean weight per beet, percentage of sucrose, and apparent purity coefficient for 10-, 20-, and 30-beet samples in each of 2 years and the average of both years—Continued

MEAN PERCENTAGE OF SUCROSE			
Year	10-beet sample	20-beet sample	30-beet sample
1933.....	16.0075	15.9635	16.0855
1934.....	16.8235	16.7270	16.7060
Average.....	16.4155	16.3452	16.3958

MEAN PURITY COEFFICIENT			
Year	10-beet sample	20-beet sample	30-beet sample
1933.....	87.6702	87.2352	87.4514
1934.....	85.0634	84.8072	85.0843
Average.....	86.3668	86.0212	86.2678

The average weights per beet for the three sample sizes were approximately on the same level, indicating that errors due to selection of larger or smaller beets for the different samples had been adequately controlled. These averages assure that the method of selecting beets in the field was satisfactory for the laboratory samples. The average percentages of sucrose and purity coefficient were sufficiently similar for both years to meet the needs of this study.

The mean squares from the analyses of variance for 10-, 20-, and 30-beet samples are given in table 2. The mean squares for weight of beets, percentage of sucrose, and purity coefficient are designated as  $w^2$ ,  $s^2$ , and  $p^2$ , respectively, and the mean products by  $ws$ ,  $wp$ , and  $sp$ .

TABLE 2.—Mean squares and mean products of weight per beet (in pounds), percentage of sucrose, and purity coefficient for 10-, 20-, and 30-beet samples in 1933 and 1934

10-BEET SAMPLES								
Year	Apportionment of variation	Degrees of freedom <sup>1</sup>	Mean square <sup>2</sup>			Mean product <sup>3</sup>		
			$w^2$	$s^2$	$p^2$	$ws$	$wp$	$sp$
1933.....	Plots within blocks.....	45	2.7327	1.3847	13.0693	-0.6102	-0.5054	2.5657
	Samples within plots.....	50	1.4697	.5046	7.4557	-.1128	.0146	.9847
	Duplicates within samples..	100	.1834	.0790	1.8304	-.0168	-.0315	.1585
1934.....	Plots within blocks.....	45	1.0995	1.2684	4.3748	-.3604	-.5739	1.3374
	Samples within plots.....	50	.3974	.1928	1.3781	-.0278	-.1999	.3353
	Duplicates within samples..	100	.0443	.0338	1.0795	-.0010	.0093	.1042
Average of 1933 and 1934.	Plots within blocks.....	90	1.9161	1.3266	8.7220	-.4853	-.5396	1.9516
	Samples within plots.....	100	.9336	.3487	4.4169	-.0703	-.0926	.6600
	Duplicates within samples..	200	.1138	.0564	1.4550	-.0089	-.0111	.1314

20-BEET SAMPLES								
Year	Apportionment of variation	Degrees of freedom <sup>1</sup>	Mean square <sup>2</sup>			Mean product <sup>3</sup>		
			$w^2$	$s^2$	$p^2$	$ws$	$wp$	$sp$
1933.....	Plots within blocks.....	45	6.3748	0.9976	19.9208	-0.3807	-0.1758	3.3073
	Samples within plots.....	50	4.5531	.3024	4.0739	-.5786	-.3815	.5763
	Duplicates within samples..	100	.4242	.0570	1.4132	-.0050	-.0516	.1141
1934.....	Plots within blocks.....	45	1.7490	.7229	4.2522	.0275	.3943	.9005
	Samples within plots.....	50	2.2546	.3878	1.6592	-.2072	-.1746	.1489
	Duplicates within samples..	100	.1630	.0404	1.0005	-.0034	-.0022	.1122
Average of 1933 and 1934.	Plots within blocks.....	90	4.0619	.8602	12.0865	-.1766	.1092	2.1039
	Samples within plots.....	100	3.4038	.3451	2.8666	-.3929	-.2680	.3626
	Duplicates within samples..	200	.2936	.0487	1.2068	-.0045	-.0269	.1132

<sup>1</sup> The degrees of freedom due to variation between blocks in each analysis are not shown.

<sup>2</sup> The mean squares for weight of beets, percentage of sucrose, and purity coefficient are designated as  $w^2$ ,  $s^2$ , and  $p^2$ , respectively.

<sup>3</sup> The mean products of weight  $\times$  sucrose, weight  $\times$  purity, and sucrose  $\times$  purity are designated as  $ws$ ,  $wp$ , and  $sp$ , respectively.

TABLE 2.—Mean squares and mean products of weight per beet (in pounds), percentage of sucrose, and purity coefficient for 10-, 20-, and 30-beet samples in 1933 and 1934.—Continued

## 30-BEET SAMPLES

Year	Apportionment of variation	Degrees of freedom	Mean square			Mean product		
			$w^2$	$s^2$	$p^2$	$ws$	$wp$	$sp$
1933.....	{Plots within blocks.....	45	7.2176	0.6807	18.9185	—0.1119	1.3945	2.7406
	{Samples within plots.....	50	5.5288	.4008	6.9691	— .3122	— .6615	.9022
	{Duplicates within samples.....	100	.6376	.0782	1.9378	— .0001	— .0973	.2134
1934.....	{Plots within blocks.....	45	6.5908	.7684	2.1113	— .5597	— .0578	.5797
	{Samples within plots.....	50	2.5640	.1361	1.7488	— .0344	— .1893	.1257
	{Duplicates within samples.....	100	.3226	.0333	.7846	— .0199	— .0902	.1067
Average of 1933 and 1934.	{Plots within blocks.....	90	6.9042	.7246	10.5149	— .3358	.6684	1.6602
	{Samples within plots.....	100	4.0464	.2684	4.3590	— .1733	— .2311	.5140
	{Duplicates within samples.....	200	.4801	.0558	1.3612	— .0060	.0036	.1600

The mean squares for variability between blocks and for total variability are not given in table 2 since they are not needed in the present study.

The beets were smaller in size in 1934 than in 1933 (table 1), and from the data in table 2 it appears that the variability was correspondingly reduced. The variance for weight of beets between samples from the same plots was less than one-half as great in 1934 as in 1933. The variability in weight between halves of the same samples (duplicates) also was less than one-half as great in 1934 as in 1933. The variability in weight is of interest insofar as the weight of beets has a bearing on the percentage of sucrose and purity coefficient.

The variance between duplicates within samples for percentage of sucrose was less than one-half as great in 1934 as in 1933 for the 10- and 30-beet samples, with a smaller reduction in the 20-beet samples. The average laboratory-error<sup>4</sup> variances for all samples were for 1933, 0.0714; for 1934, 0.0358; and for the 2 years, 0.0536. The corresponding standard errors of a single analysis would be, therefore, 0.2672, 0.1892, and 0.2315. This reduction probably is due to greater precision in the laboratory in 1934, since the variability between duplicates must be due to laboratory errors alone.

As an average of both years, it cannot be said that the variance between duplicates for percentage of sucrose (laboratory-error variance) was different for the 10-, 20-, and 30-beet samples, the variances being 0.0564, 0.0487, and 0.0558, respectively. Since there seems to be no difference between the laboratory variances for 10-, 20-, and 30-beet samples, it may be concluded that inaccuracies due to splitting the beets into halves, rasping, and mixing the larger mass of pulp from the 30-beet samples as compared with the smaller samples were not significantly different in the three sample sizes.

The laboratory-error variance for purity coefficient, like that for percentage of sucrose, was much lower in 1934 than in 1933. The average laboratory-error variances for all samples were for 1933, 1.7271; for 1934, 0.9549; and for the 2 years, 1.3410. The corresponding standard errors of a single analysis were 1.3142, 0.9772, and 1.1580. The average laboratory-error variances for the 2 years were 1.4550, 1.2068,

<sup>4</sup> The term "laboratory error" is used to designate all possible sources of error that can occur in the laboratory.

and 1.3612 for the 10-, 20-, and 30-beet samples, respectively. There was no consistent variation in the variances with increase in sample size.

It is of interest to compare the relative magnitude of the laboratory-error variances for percentage of sucrose and purity coefficient. The average variance for percentage of sucrose for all samples was 0.0536, while that for purity coefficient was 1.3410; hence the variance of purity coefficient was 25 times as great as that for percentage of sucrose. Table 1 shows that the average percentage of sucrose for all samples was 16.3855, while the average purity coefficient was 86.2186, the latter being 5.26 times as great as the former. It is apparent, therefore, that the relative variance (variance divided by the squared mean) was very nearly the same for both percentage of sucrose and purity coefficient. The relative variance for percentage of sucrose was  $.0536 \div (16.3855)^2$ , or 0.00020, while that for purity coefficient was  $1.3410 \div (86.2186)^2$ , or 0.00018. The standard errors for laboratory errors in percentage of the mean were 1.41 and 1.34 for percentage of sucrose and purity coefficient, respectively.

The mean products (table 2) for weight of beets and percentage of sucrose are negative, with one exception, and indicate a small negative correlation. The mean products for weight and purity coefficient were more often negative than positive, and very small. However, the mean products of percentage of sucrose and purity coefficient were positive and indicate positive correlation between these two variables. The significance of the correlation coefficients to be derived from the mean products and mean squares will be discussed in greater detail later.

The variance due to variability between duplicates within samples is due to errors in the laboratory method. The variance due to variability between samples within plots is due to laboratory errors plus the true variance between the individual samples from the same plots. The latter is due to genetic and environmental factors affecting the weight, percentage of sucrose, and purity coefficient of the individual beets.

Table 3 brings together data, taken from table 2, on the two variances for percentages of sucrose for the three sample sizes.

TABLE 3.—Mean squares for percentages of sucrose and for purity coefficient of 10-, 20-, and 30-beet samples taken in 1933 and 1934

Year	Apportionment of variation	De- grees of free- dom	Mean square for percentage of su- crose in samples having indicated number of beets			Mean square for pu- rity coefficient in samples having in- dicated number of beets		
			10	20	30	10	20	30
1933-----	{Samples within plots.....	50	0.5046	0.3024	0.4008	7.4557	4.0739	6.9691
	{Duplicates within samples..	100	.0790	.0570	.0782	1.8304	1.4132	1.9378
1934-----	{Samples within plots.....	50	.1928	.3878	.1361	1.3781	1.6592	1.7488
	{Duplicates within samples..	100	.0338	.0404	.0333	1.0795	1.0005	.7846
Average for 1933 and 1934.	{Samples within plots.....	100	.3487	.3451	.2684	4.4169	2.8666	4.3590
	{Duplicates within samples..	200	.0564	.0487	.0558	1.4550	1.2068	1.3612

While the laboratory-error variances for percentage of sucrose were reduced in 1934 as compared with those in 1933, the ratio of the laboratory-error variance to the variance between samples within plots was relatively similar in both years. Consequently, the interpretation of these data will be based on the averages of both years.

In order to estimate the expected variance for a given number of samples from a plot and a given number of laboratory analyses per sample the formula  $K = \frac{p}{n} + \frac{m}{Nn}$  may be used,<sup>5</sup> where

$K$ =required variance per plot,

$N$ =number of samples per plot,

$n$ =number of laboratory analyses per sample,

$m$ =variance due to laboratory errors (between duplicates within samples),

$p$ =variance between samples within plots due to genetic or environmental factors; i. e., variance between samples within plots minus variance between duplicates within samples expressed on a sample basis.

Table 4 shows the average values of  $p$  and  $m$  for 1933 and 1934.

The values of  $m$  are taken directly from table 3, and the values of  $p$  are computed from the same table. The value of  $p$  for the 10-beet samples, for example, is  $\frac{0.3487 - 0.0564}{2} = 0.1462$ . To convert this

variance from the basis of a single laboratory analysis to a sample basis it is necessary to divide by 2, since two analyses were made on each sample. The variance of a single plot is seen to be made up of two components: (1) The laboratory-error variance and (2) the variance due to the true variability between samples. Both contribute to the error of the plot.

TABLE 4.—Variance  $p^1$  and variance  $m^2$  in percentage of sucrose of 10-, 20-, and 30-beet samples for the 2-year averages for 1933 and 1934

Apportionment of variance	10-beet samples	20-beet samples	30-beet samples
$p$ .....	0.1462	0.1482	0.1063
$m$ .....	.0564	.0487	.0558

<sup>1</sup>  $p$ =variance between samples within plots due to genetic or environmental factors, i. e., variance between samples within plots minus variance between duplicates within samples expressed on a sample basis.

<sup>2</sup>  $m$ =variance due to laboratory errors (between duplicates within samples).

As pointed out previously, the laboratory-error variances ( $m$ ) do not seem to vary with the size of the sample. The estimated variances for the true variability ( $p$ ) between the 10-, 20-, and 30-beet samples were 0.1462, 0.1482, and 0.1063, respectively. These variances would be expected, theoretically, to be inversely proportional to the size of the sample, i. e., 10-, 20-, and 30-beet samples, as the degrees of freedom increase without limit. The values of  $p$  for the 20- and 30-beet samples are approximately proportional to the inverse ratio of the number of beets in the sample, but the value of  $p$  for the 10-beet samples is lower than expected.

<sup>5</sup> IMMER, F. R. A STUDY OF SAMPLING TECHNIC WITH SUGAR BEETS Jour Agr Research 44 633-647 1932.

Increasing the number of laboratory analyses per sample or reducing the laboratory error by more careful technique reduces the variance  $m$ . Likewise, increasing the number of samples of beets for sucrose analysis from each plot reduces the variance  $p$ . The experimental error of the individual plots is made up of both  $m$  and  $p$ .

If one 20-beet sample is taken from a plot and duplicate sugar analyses are made on the sample, the variance for the plot (using the averages for both years) is

$$K = 0.1482 + \frac{0.0487}{2} = 0.1726$$

If the two 20-beet samples are taken from a plot and only one laboratory analysis is made on each sample, the plot variance is

$$K = \frac{0.1482}{2} + \frac{0.0487}{2} = 0.0984$$

Thus it is seen that if the total number of analyses is held constant, increasing the number of samples from a plot decreases the error of the experiment more rapidly than does increasing the number of laboratory analyses per sample. If but one 20-beet sample is taken from each plot and one analysis is made, the variance is

$$K = 0.1482 + 0.0487 = 0.1969$$

Even though the laboratory variance were reduced to zero, the plot variance ( $K$ ) would still remain at 0.1482, or 75 percent as great as at present. Thus, increasing the precision in the laboratory can affect only 25 percent of the plot variance obtained in this study.

Greater precision in making the laboratory analyses can be expected to reduce the variance  $m$ , but will have no effect on the value of  $p$ . While the laboratory errors are probably greater than could be obtained by more careful work by a more adequately trained technician, they are relatively small as compared with the large errors due to the variability between the individual beets.

In table 3 are given the variances of purity coefficients for samples within plots and duplicates within samples for the 10-, 20-, and 30-beet samples. The laboratory error as measured by variance between duplicates within samples, for the average of all three sample sizes, was only 55 percent as great in 1934 as in 1933. The variance for percentage of sucrose in 1934 was 61 percent as great as in 1933. Thus it appears that the reduction in the error in purity coefficient followed closely the reduction in the error in percentage of sucrose. It is the reduction in variance between samples within plots which is more striking, the variance in 1934 being only 74 percent as great as that in 1933. Growing conditions in the autumn of 1933 were normal, whereas in 1934 a very severe and protracted drought greatly delayed growth and reduced the size of the beets. It was shown in table 2 that the beets were more uniform in size and in purity coefficient in 1934 than in 1933. Therefore, it seems reasonable to assume that this reduction in variability in purity coefficient probably was associated to a considerable degree with climatic conditions conducive to greater uniformity between beets.

As an average of both years, the variance  $m$  (duplicates within samples) was 1.4550, 1.2068, and 1.3612 for the 10-, 20-, and 30-beet samples, respectively. The variance was lowest for the 20-beet samples but this probably was due to error of random sampling. It appears that the laboratory-error variances for purity coefficient did not vary significantly with size of sample. This fact would follow logically after the conclusions drawn for percentage of sucrose.

The values of variance  $p$  for the 10-, 20-, and 30-beet samples, as an average of both years, were 1.4810, 0.8299, and 1.4989, respectively. Thus, the estimated true variance between samples (apart from laboratory errors) was the lowest for the 20-beet samples. These values of variance  $p$  were essentially the same for the 10- and 30-beet samples, however.

As an average of both years, the ratio of values of the variance  $p$  to variance  $m$  for percentage of sucrose varied from 2:1 to 3:1 with different sizes of samples. The same ratio for purity coefficient varied from 1:1 to 0.7:1. From this it is apparent that the laboratory errors are of greater importance for purity coefficient than for percentage of sucrose. Therefore, improvements in laboratory technique leading to lower laboratory errors are relatively more important in reducing the experimental error for purity coefficient than for percentage of sucrose. Any reduction in the laboratory error for percentage of sucrose would reduce the error for purity coefficient as well, since the saccharimeter reading is used in the calculation of purity coefficient.

Since the variances  $p$  and  $m$ , for the 2-year average of the 20-beet samples, were 0.8299 and 1.2068, respectively, a single sample per plot and but one laboratory analysis would give a variance of  $K=0.8299+1.2068$ , or 2.0367. The laboratory-error variance (1.2068) would be 59 percent of this plot error. In the case of percentage of sucrose, the laboratory-error variance was but 25 percent of the plot variance.

The laboratory errors for purity coefficient were relatively more important in 1934 than in 1933. Using the 20-beet samples as an example, the laboratory-error variances were 52 and 75 percent of the plot variances for a single analysis on a single sample in 1933 and 1934, respectively.

Since the relative variances (variance divided by squared mean) of laboratory error for percentage of sucrose and purity coefficient were very similar and the estimated variances due to the true variability between beets (variance  $p$ ) were proportionately much greater than variances  $m$  for percentage of sucrose than for purity coefficient, it appears clear that the beets varied considerably less in purity coefficient than they did in percentage of sucrose. This was true particularly in 1934.

When the total number of laboratory tests was held constant, making several analyses per sample reduced only variance  $m$  whereas analyzing several samples with only one laboratory analysis per sample reduced both variance  $m$  and variance  $p$ .

From the foregoing it is apparent that it is preferable to analyze several samples from a plot, with only one analysis of each sample, than to make several analyses per sample.



In table 5 are given the coefficients of correlation for (1) weight of beets with percentage of sucrose, (2) weight with purity coefficient, and (3) percentage of sucrose with purity coefficient. The coefficients were calculated directly from the mean products and mean squares of table 2.

TABLE 5.—Coefficients of correlation for weight and percentage of sucrose, weight, and purity coefficient, and percentage of sucrose and purity coefficient in 10-, 20-, and 30-beet samples taken in 1933 and 1934

10-BEET SAMPLES					
Year	Apportionment of variation	Degrees of freedom	Correlation coefficient of— <sup>1</sup>		
			<i>w</i> and <i>s</i>	<i>w</i> and <i>p</i>	<i>s</i> and <i>p</i>
1933.....	{Plots within blocks.....	45	—0.3137	—0.0846	0.6031
	{Samples within plots.....	50	—0.1310	—0.044	.5077
	{Duplicates within samples.....	100	—0.1366	—0.054	.4168
1934.....	{Plots within blocks.....	45	—0.3052	—0.2617	.5677
	{Samples within plots.....	50	—0.1004	—0.2701	.6505
	{Duplicates within samples.....	100	—0.0258	.0425	.5455
Average of 1933 and 1934...	{Plots within blocks.....	90	—0.3044	—0.1320	.5737
	{Samples within plots.....	100	—0.1232	—0.4556	.5318
	{Duplicates within samples.....	200	—0.1110	—0.0273	.4587
20-BEET SAMPLES					
1933.....	{Plots within blocks.....	45	—0.1510	—0.0156	0.7419
	{Samples within plots.....	50	—0.4931	—0.0839	.5195
	{Duplicates within samples.....	100	—0.0485	—0.0666	.4020
1934.....	{Plots within blocks.....	45	—0.0245	.1446	.5136
	{Samples within plots.....	50	—0.2216	—0.0903	.1856
	{Duplicates within samples.....	100	—0.0419	—0.0054	.5581
Average of 1933 and 1934...	{Plots within blocks.....	90	—0.0945	.0156	.6525
	{Samples within plots.....	100	—0.3625	—0.0858	.3646
	{Duplicates within samples.....	200	—0.0376	—0.0452	.4669
30-BEET SAMPLES					
1933.....	{Plots within blocks.....	45	—0.0505	0.1193	0.7637
	{Samples within plots.....	50	—0.2097	—0.1066	.5398
	{Duplicates within samples.....	100	—0.0004	.0875	.5482
1934.....	{Plots within blocks.....	45	—0.2487	—0.0155	.4551
	{Samples within plots.....	50	—0.0582	.0941	.2576
	{Duplicates within samples.....	100	—0.1148	—0.1793	.6601
Average of 1933 and 1934...	{Plots within blocks.....	90	—0.1501	.0784	.6015
	{Samples within plots.....	100	—0.1603	—0.0550	.4752
	{Duplicates within samples.....	200	—0.0367	.0045	.5806

<sup>1</sup> *w*=weight; *s*=percentage of sucrose; *p*=purity coefficient.

In the notation previously employed for designating the mean squares and mean products, the coefficient of correlation between weight of beets and percentage of sucrose would be

$$r = \frac{S(ws)}{\sqrt{S(w^2)} \sqrt{S(s^2)}}$$

The correlation coefficient for variability between duplicates within 10-beet samples in 1933, for example, as calculated from table 2, would be

$$\frac{-0.0168}{\sqrt{0.1834} \sqrt{0.0790}} = -0.1396$$

In testing the significance of these correlation coefficients we may use the 5-percent level of significance to test whether the observed coefficients could have arisen by chance from an uncorrelated population.<sup>6</sup> To exceed the 5-percent level of significance the correlation coefficients would need to be greater than the values given below, for the degrees of freedom in table 5.

<i>Degrees of freedom</i>	<i>Correlation coefficients at 5-percent point</i>
45.....	0. 2905
50.....	. 2757
90.....	. 2061
100.....	. 1955
200.....	. 1384

The correlation coefficients for weight of beets and percentage of sucrose between duplicates within samples were all negative, though nonsignificant. It is clear, therefore, that the slight differences between the weights of the two halves of the split sample of beets (duplicates) did not significantly affect the percentage of sucrose. Thus, splitting of the beets prior to rasping the pulp for analysis was accomplished with sufficient accuracy to prevent this from being a source of detectable error in the sucrose analyses. While an inherent negative correlation between weight of beets and percentage of sucrose between samples within plots is evident, only in one instance, that of the 20-beet samples in 1933, was the correlation coefficient significant. This coefficient made the correlation for the average of both years significant for that comparison. It appears that variability in weight between samples from the same plots was not sufficiently large to affect appreciably the percentage of sucrose.

The purity coefficient was not significantly correlated with weight of beets for any of the comparisons made.

The coefficients of correlation between percentage of sucrose and purity coefficient were all positive. Only 2 coefficients out of 27 calculated failed to reach the 5-percent level of significance, namely, those for samples within plots in 1934 for both the 20- and 30-beet samples.

As an average for both years, the correlation between percentage of sucrose and purity coefficient, for variability between duplicates within samples, was expressed in coefficients of 0.4587, 0.4669, and 0.5806 for the 10-, 20-, and 30-beet samples, respectively. These coefficients are highly significant and it can be concluded that the purity coefficient was closely associated with percentage of sucrose.

Calculating the regression of purity coefficient on percentage of sucrose, for variability between duplicates within samples, it is found that the regression coefficients from the averages of both years were 2.3298, 2.3244, and 2.8674 for the 10-, 20-, and 30-beet samples, respectively. These coefficients were computed from the mean products and mean squares found in the appropriate rows in table 2. From the regression coefficients it can, therefore, be stated that as percentage of sucrose increased by 1 percent the purity coefficient increased by 2.33, 2.32, and 2.87 percent in the 10-, 20-, and 30-beet samples, respectively.

<sup>6</sup> FISHER, R. A. (See footnote 3)

It is only the portion of the variability in purity coefficient not associated with percentage of sucrose which is of appreciable value. The percentage of the variability in purity coefficient (as measured by sums of squares) which is associated with percentage of sucrose will be given by  $r^2$ . Squaring the coefficient of correlation (as an average of both years) between purity coefficient and percentage of sucrose, for variability between duplicates within samples, it may be said that 21, 22, and 34 percent of the sums of squares for purity coefficient, in the 10-, 20-, and 30-beet samples, respectively, was associated with variability in percentage of sucrose. Roughly one-fourth of the variability in purity coefficient between duplicates was dependent on its inherent association with percentage of sucrose, leaving 75 percent independent of variability for percentage of sucrose.

#### SUMMARY

A study was made of the errors of routine analysis for percentage of sucrose and purity coefficient in sugar beets taken from field experiments. Comparative analyses were made on 10-, 20-, and 30-beet samples in each of 2 years.

The laboratory errors between 10-, 20-, and 30-beet samples did not vary significantly for either percentage of sucrose or purity coefficient.

The laboratory error was a proportionately greater part of the plot error for purity coefficient than for percentage of sucrose. Increasing the number of samples analyzed per plot would reduce the plot variance more rapidly than increasing the number of analyses per sample.

A negative, though nonsignificant, correlation coefficient for variability between duplicate analyses of the same samples was obtained between weight of beets and percentage of sucrose or purity coefficient.

The correlation between percentage of sucrose and purity coefficient was positive and highly significant. As the percentage of sucrose increased by 1 percent the purity coefficient increased by 2.33, 2.32, and 2.87 percent in the 10-, 20-, and 30-beet samples, respectively. Approximately one-fourth of the variability in purity coefficient, as measured by the differences between duplicate samples, could be ascribed to its inherent association with percentage of sucrose.



# AVAILABILITY OF THE COPPER OF BORDEAUX MIXTURE RESIDUES AND ITS ABSORPTION BY THE CONIDIA OF *SCLEROTINIA FRUCTICOLA*<sup>1</sup>

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## INTRODUCTION

The present paper deals with the deposit left by the drying of a bordeaux mixture spray and its effect on the conidia of *Sclerotinia fructicola* (Wint.) Rehm. This residue consists of the sediment or precipitate and the materials that were dissolved in the water that evaporated. It has been established by previous workers that a fresh residue usually contains calcium hydroxide, calcium sulphate, calcium carbonate, basic copper sulphate, and copper hydroxide. That it is very slightly soluble, as indicated by its resistance to washing by rains, has been established by studies of the copper compounds it contains.

The work reported herein was chiefly concerned with the toxicity of the residues after aging and weathering, the latter term including the effects of rain, dew, atmospheric gases, and plant excretions.

## REVIEW OF LITERATURE

Studies of the physical and chemical properties of bordeaux mixture (7, 22, 24)<sup>3</sup> have shown that the copper is in the form of basic sulphates, which investigators have assumed become slightly soluble when acted upon by carbon dioxide of the air or by spore and host excretions.

A large literature has been built up around the above-named hypotheses, which have been well discussed by Martin (21) and McCallan (19). According to these hypotheses, the toxicity of the basic sulphates of copper is due to the liberation of soluble copper after the materials have been deposited upon the host plants, and not to the soluble ions and soluble complexes inherent in the deposits themselves.

Whetzel and McCallan (29, p. 3) summarized the published information on the solubility of bordeaux mixture in these words:

It is generally recognized, however, that certain of our standard fungicides, notably bordeaux mixture and sulfur dust, are not sufficiently soluble in pure water to exercise their fungicidal properties.

Although it has been the accepted theory that the precipitates of bordeaux mixture are not in themselves sufficiently soluble to exert a toxic influence, and a considerable amount of research has

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<sup>2</sup> The writers wish to express their appreciation to J. W. Roberts, of this Division, for his helpful suggestions during the course of this investigation, and to other members of the Bureau of Plant Industry and to members of the Bureau of Chemistry and Soils for their criticisms in the preparation of the manuscript.

<sup>3</sup> Reference is made by number (*italic*) to Literature Cited, p. 532.

been carried out to determine how and why these precipitates become soluble, it is only recently that we have come to an understanding of some of the principles involved. Although it has been recognized by Clark (10), Crandall (11), Barker and Gimingham (4), McCallan (19), Lutman (17), and many others that bordeaux mixture precipitates are toxic to fungus spores from the time they are applied, it still remains to be proved just how that toxic effect is produced.

The study of bordeaux mixture must take account of its chemical composition and properties, and for this reason a review of the pertinent knowledge on these subjects is presented. The chemical composition of bordeaux mixture is known to be quite complex. One reason for this is found in the wide range of proportions and concentrations of the solution of copper sulphate and the solution or suspension of calcium hydroxide that are stirred together to make bordeaux mixture.

Pickering (24) concluded that the proportion of lime should not exceed that necessary to react with all the copper sulphate. He found this to be three-fourths of a chemically equivalent weight, or a trifle more than 1 part by weight of hydrated lime to 5 parts of copper sulphate crystals. This fact and his studies of the precipitate showed that the copper compound formed by the reaction was the basic sulphate  $\text{CuSO}_4 \cdot 3\text{Cu}(\text{OH})_2$ , the same that had been described long before by Proust (26, p. 34) as the result of the prolonged reaction of copper hydroxide with copper sulphate. According to Posnjak and Tunell (25), extensive studies of copper compounds have by now led most of the interested authorities to conclude that at temperatures between  $0^\circ$  and  $40^\circ$  C. and near the neutral point this compound is the only one formed. Pickering's restriction of the lime kept the alkalinity of his preparation in this same range. An alkalinity greater than 0.05 normal (pH 12.5) cannot be attained with lime because of its small solubility. Thus certain basic compounds of copper that have been shown to be formed in more alkaline solutions are formed only slowly and incompletely in calcium hydroxide solutions.

Injuries to host plants have compelled the use of much greater proportions of lime than those recommended by Pickering (24); in fact, the weight of lime taken at present is more likely to be at least equal to the weight of the copper sulphate than to be only a fifth of it. The sediment of these mixtures contains calcium hydroxide in addition to the sulphate, for more is present than will dissolve. This circumstance makes the basicity of the insoluble copper compounds difficult to estimate, and accordingly divergent results have been reported. These results have led to suggestions that a number of basic compounds of copper and double compounds of lime and copper are formed. However, Martin (22) has presented evidence of a slow transformation of the basic sulphate first formed to the hydroxide, and offers this as an explanation of the divergent results.

Since the immediate effect of bordeaux mixture in the control of fungi (4, 10, 11, 17, 19) and obvious damage to certain host plants (3) cannot be adequately explained except by assuming that some of the copper passes into solution, the copper compounds of the residue cannot be considered strictly insoluble. In one of the earliest papers on bordeaux mixture, Millardet and Gayon (23) suggest an inherent solubility, and more recently Hockenyos (14) and Ribéreau-Gayon

(27) found that this solubility measures several parts per million. In the work presented in this paper the writers have found that the copper of bordeaux mixture dissolves in water at the rate of about 4 parts per million. The behavior of bordeaux mixture as reported herein indicates that something different from the inherent solubility of these copper compounds is responsible for the fact that the copper is readily taken up from certain residues by fungus spores. These results agree better with the theory that there is present a varying quantity of a certain sort of copper compound, not necessarily ionic, which is subject to absorption by the spores. For this property the term "availability" is suggested to distinguish it from solubility, which means the quantity of the residue constituent that will dissolve without first undergoing change.

That the solubility of unaltered bordeaux mixture residues in large quantities of water does not yield sufficiently high concentrations of copper to be harmful to the test spores has been proved by a number of investigators (4, 19, 21, 28); but contact with the particles of the same residues in small quantities of water has killed some of the same spores with little delay. It is also well known that the shorter the distance between a particle of residue and a spore the sooner toxicity becomes evident (4).

The literature discussed so far has dealt exclusively with freshly made precipitates or with fresh residues. It is obvious that practical work in the field has to do with residues in this condition for only a relatively short time and that most of their usefulness comes after a certain period of aging and exposure to weather. The resultant effects upon the spray residues need to be understood, particularly in establishing bases of comparison in the development of new fungicides. Recently Branas and Dulac (5) reported the results of their studies on copper dissolved from residues that were washed with solutions made up to imitate rain water; however, the residues were filtered off and were not studied.

#### MATERIALS AND METHODS

Residues resulting from bordeaux mixture sprayed upon microscope cover slips were exposed to natural weathering in the orchard and then brought into the laboratory for critical tests, both as to chemical changes and as to toxicity to organisms. Tests were also carried out with residues that had been aged in the laboratory.

Conidia of *Sclerotinia fructicola* were used in this work. The quantity of similar work done with this organism attests its suitability. These conidia are large, hyaline, easily stained, and have large vacuoles and well-defined granules. Germinability is high and dependable. For the most part the conidia were from 10- to 16-day-old cultures grown upon nutrient potato hard agar. Occasionally the conidia were obtained from growths upon sterile string bean and from rotting peaches and plums.

The precautions given by McCallan (18) for germination tests were strictly observed. Suspensions of the conidia were prepared so that they contained about 30 cells to a low-power field ( $\times 125$ ).

The tests for toxicity were carried out by placing the conidia of *Sclerotinia fructicola* in drops of water or dilute nutrient solutions upon

the residues, the cover slip being inverted and used as the cover of a Van Tieghem cell. All tests were made in duplicate.

Thick (no. 2) cover slips were used to reduce breakage. The 24- by 50-mm size was chosen because two tests could be made with each slip by breaking it into halves. The slips were cleaned, numbered on the back, and laid upon paper towels on a horizontal surface, none touching another slip. They were then sprayed by means of a hand atomizer until each was covered with a complete layer of spray liquid, and dried. Averaging a number of determinations of total copper (Cu) upon slips prepared in this way gave 36 micrograms per square centimeter, which is in reasonable agreement with the amount found by Hockenyos and Irwin (15) and by Holland, Dunbar, and Gilligan (16) for the surface of leaves sprayed in the orchard.

The bordeaux mixture spray used in all tests contained the equivalent of 2 pounds of copper sulphate ( $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ ), 4 of hydrated lime (70 percent  $\text{CaO}$ ), and 2 of bentonite to 50 gallons of water. The bentonite was added to improve adhesiveness and was used in other tests being compared with these. For comparison, in order to evaluate the fungicidal effect of the lime, residues from similar sprays where zinc replaced copper were used. The zinc compounds seemed to be without permanent effect upon the germination of the spores, and the alkalinity of the lime would be neutralized in about the same way as in bordeaux mixture. While it may be argued that a calculated quantity of sulphuric acid would be a more defensible check than zinc sulphate, the results seemed to prove the zinc-lime spray satisfactory. Its use was convenient because it gave residues that were toxic only for a day or so and apparently indifferent afterward. This no doubt was due to the loss in the initial alkalinity of the fresh precipitate.

After being dried, the slips were either stored in the laboratory in a microscope-slide box modified to contain them or hung up among the leaves in the orchard in a special holder designed for this purpose (fig. 1). This holder was made by fastening a piece of wood to one

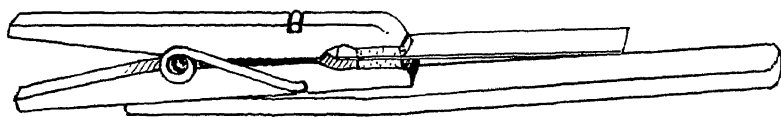


FIGURE 1.—Holder for exposing sprayed slips in orchards.

side of a clamp clothespin to protect the cover slip from breakage by wind. The jaws were surfaced with cork. Unless a means can be found to make a satisfactory germination test upon a leaf, this seems to represent the nearest possible approach to exposing residues to the environmental influences that affect residues upon the plant. Unsprayed slips were sometimes hung in the orchard and were sprayed when the tree was sprayed. Work reported in this paper suggests that studies of this kind offer information not to be had by aging the residues in any other known way.

Initial germination was found to be somewhat more vigorous and constant when nutrient materials were added to distilled water, and such a medium appeared to be more suitable than distilled water alone. Clumping of the conidia, or the tendency of some of the cells to resist



wetting and, therefore, submersion, frequently occurred in most of the dilute nutrient media used. This is a distinct disadvantage, since some of the cells do not have an opportunity to absorb the nutrient media and germination may be delayed. To obviate this, recourse was had to a material in no way injurious in itself, namely, coenzyme R, which has the property of wetting each spore so that the cells have the same electrical charge. Germination was found not to be particularly hastened by this method, but it was noted that a greater number of conidia began their activity at one time. This, no doubt, is a consequence of each cell having the same opportunity of absorbing nutrients. The original paper on coenzyme R, by Allison, Hoover, and Burk (1), described it as a respiratory coenzyme. The present work indicates that it also produces a change in the surface tension of the cell walls of the organisms, allowing this surface to become wet with the suspensoid containing the nutrient materials. In this manner the uncertain effects of clumping have been eliminated, and it is felt that the work is more standardized because of it. When the spore suspension was applied to a surface of spray residue, the liquid always showed a strong tendency to spread out among the particles of the spray residue. This, no doubt, was caused by the ability of the suspensoid to wet the particles of spray residue thoroughly. When such a film was placed in the saturated atmosphere of a Van Tieghem cell it remained so thin that germination was interfered with, apparently because of a lack of "free" water. Accordingly, the residue outside of a circle 6 or 7 mm in diameter was scraped away from the cover slip; with this precaution satisfactory germination tests were obtained.

After a small drop of the suspension delivered by a microloop had been transferred to the prepared cover slip containing the residues, the cover slip was inverted over a Van Tieghem cell and incubated at room temperature for a period of time varying according to the object of the test.

After every germination test some of the conidia were transferred from the cover slip to an agar slant to find out whether they had been killed or whether their germination had been merely inhibited. McCallan (19) and McCallan and Wilcoxon (20) do not mention this step, but it is important for the true evaluation of a fungicide. It is known that toxic materials in less than lethal doses or minor deficiencies in the environment may prevent spores from germinating. But failure of germination alone, without plasmolysis, is not a conclusive proof of death, for if the spore is removed from the unfavorable environment and the toxic material absorbed is given an opportunity to diffuse into a medium containing practically none of it, germination may take place. The implications of such a result in the field use of sprays is obvious. Residues from which spores germinated after such a transfer have been designated "inhibitory."

It was inevitable that the investigator should form an impression of the quantity and tenacity of the residue while scraping off the unwanted portion. This is presented with the data on germination. "Excellent" was used to describe a residue that seemed as heavy as when freshly prepared. Other terms were used with their usual connotations, "fair" meaning a barely continuous film over the glass and "poor" a residue with obvious defects.

A colorimetric method was employed to detect soluble copper on the bordeaux mixture residues and in the solution drops on the residues. During the last 10 years a number of sensitive reagents for copper have been developed (8, 9, 12, 13). The one used in this work is the sodium salt of nitroso-1, 8-dihydroxynaphthalene-3, 6-disulphonic acid, discovered by Brenner (6), and adapted for the micro-determination of copper by Cherbuliez and Ansbacher (9). The concentrated stock solution of this compound, called "the chromotropic reagent" by Ansbacher, Remington, and Culp (2), diluted with an equal volume of distilled water, is to be understood in this work where treatment with chromotropic solution is mentioned. Other chemicals scarcely affect the formation of the diagnostic colored compound formed on the addition of this solution to substances containing soluble copper, and the simplicity of the operations required commended it for the present purpose. The color is an intense purple, and because it is different from other colors generally encountered in plant cells, it is useful for the microscopic recognition of copper inside cells.

Direct application of a drop of chromotropic solution to a fresh, highly toxic copper-bearing residue invariably gives the characteristic purple color which is indicative of the soluble copper inherent in the residue. Generally a much deeper color is obtained if the residue is first treated with the fumes of nitric acid, then wet with a drop of 1-percent ammonium hydroxide and allowed to dry. There is reason to believe that this treatment disengages the copper from certain complexes and insoluble compounds in the residue which do not give the test before such treatment. This procedure is accordingly considered to yield a purple "spot", the intensity of which is roughly proportional to the total amount of copper present.

As has been mentioned, each 24- by 50-mm cover slip was broken into two pieces and duplicate germination tests were run. From one of these a transfer was made to an agar slant to see whether viable spores still remained. The other half was allowed to dry and was then treated with nitric acid, ammonia, and chromotropic solution, as described above, and the intensity of the resulting purple spot was recorded. Values assigned to these intensities were as follows: 4, Indistinguishable from a freshly prepared slip; 3, noticeably weaker than 4; 2, having a color roughly half as deep as that of 4; and 1, very faint.

#### CHANGES IN TOXICITY, APPEARANCE, AND TOTAL COPPER OF BORDEAUX MIXTURE RESIDUES AFTER WEATHERING

Table 1 presents one of a series of tests conducted with the methods just described. The cover slips were sprayed on August 7, 1933. Some were hung up in the fruit-disease orchard at Arlington farm, Rosslyn, Va., and removed for tests after the indicated intervals. Others were stored in a closed box in the laboratory until August 18, when they were placed in holders in the same trees. They were also tested after intervals of exposure. The total number of days of orchard or laboratory exposure and the total rainfall for each slip were recorded. The differences in effect of age without rainfall and of age combined with rainfall are shown.

TABLE 1.—Changes in toxicity, appearance, and total copper of bordeaux mixture residues after weathering

Period of exposure		Rainfall	Incubation period	Germination	Growth after transfer <sup>1</sup>	Appearance of residue	Amount of copper present <sup>2</sup>
In laboratory	In orchard						
Days	Days	Inches	Hours				
0	3	( <sup>3</sup> )	20	None.....	None.....	Excellent.....	4
0	5	1 18	24	Few buds <sup>4</sup> .....	None.....	do.....	4
0	7	1 18	24	None.....	Yes.....	do.....	4
0	11	1 97	20	Few buds.....	Yes.....	do.....	4
0	17	4 48	23	None.....	Yes.....	Very good.....	2
0	37	10. 72	48	None.....	Yes.....	do.....	2
11	( <sup>1</sup> )	( <sup>3</sup> )	20	None.....	None.....	Excellent.....	4
35	7	.98	24	None.....	Yes.....	do.....	3
35	11	1 17	24	None.....	Yes.....	do.....	4
35	18	1 17	20	Few buds.....	Yes.....	do.....	3
35	21	1. 41	48	do.....	Yes.....	Good.....	3

<sup>1</sup> Due to activity of conidia inhibited, and not killed, by the treatment.

<sup>2</sup> Total amount of copper determined by exposing the remaining residue to nitric acid fumes, neutralizing with ammonium hydroxide, and adding chromotropic solution to the remaining liquid. The figure <sup>4</sup> represents the deepest color obtained with this method.

<sup>3</sup> None.

<sup>4</sup> The word "bud" indicates the primordial germ tube or the beginning of germination

It is seen that field exposure and rainfall gradually lessened the toxicity of the spray deposits, as is usual with bordeaux mixture residues. The toxicity diminished to the point where germination was checked but the conidia were not all killed, as evidenced by the fact that germination was resumed when the environment became favorable. That age alone did not lessen toxicity is proved by the tests with the slips stored in the laboratory. From these results it may be inferred that the copper compounds that imparted the original toxicity have been diluted or washed off, leaving others that could not be dissolved sufficiently to kill all the conidia. It must be emphasized that at no time during the periods of weathering were the copper residues sufficiently free of available copper to allow free germination of the conidia. There was always sufficient available copper present to cause inhibition, at least; and that a portion of the original residue remained at the end of the weathering period was plain to the eye.

It must be added that with the residues recorded as only inhibitory in table 1 many of the conidia were actually killed. Some of the conidia showed plasmolysis of the cell contents exactly as did those killed by relatively fresh residues. From this fact one would conclude that the supply of available copper had not been evenly distributed among the conidia.

From these experiments the impression was gained that some of the copper was present in such a form as not to be available to the organisms and that the conidia have not the power to dissolve copper in this form. This was particularly evident in those experiments where the residues became scanty although the total copper present, if brought into solution by conidial secretions, should have been sufficient to be toxic to all the seeded conidia.

The data presented in table 1 do not indicate the mechanism by which the injured conidia absorbed the copper. It is shown that, irrespective of the mechanism, a point is reached in the course of

prolonged weathering of the residue where, although copper is still present, its toxicity is low compared with that in an equal quantity of unweathered residue.

Only lines 1 and 7 of table 1 relate to residues not exposed to rainfall, and hence only these are comparable with the results of Barker and Gimingham (4) and of McCallan (19). Their work consistently shows that fresh residues are directly toxic to spores, but they considered that this was due to copper brought into solution by some solvent secreted by the spores themselves. The technique used by the writers could scarcely have prevented the conidia from exerting the same dissolving action, but the results of the experiments show that on the excessively weathered residues it must have been without effect.

#### ABSORPTION OF AVAILABLE COPPER FROM SPRAY RESIDUES BY CONIDIA OF *SCLEROTINIA FRUCTICOLA*

Experience in the application of the chromotropic solution to spray residues suggested its use to demonstrate the presence of copper inside conidia that had been exposed to copper ions. Trials demonstrated that the chromotropic solution diffused easily into conidia, showing its original yellow color when no copper was present and imparting a uniform purple color to the interior of those shown by collateral tests to have been affected by copper. So far efforts to demonstrate localized deposits of copper in the affected cells have failed.

The experiments were set up in exactly the same manner as described in the foregoing section, and the conidia were allowed to germinate over a 24-hour period. At the end of the incubation period a microloop of the chromotropic solution was added to the conidial suspension in contact with the bordeaux residues. The treatment caused a deep purple color to appear upon the surface of the spray particles and in the solution surrounding the particles. When conidia killed by the copper in the residues were washed free of the treated residue and residue solution, microscopic examination showed the protoplasm stained a deep purple. When residues have been reduced in toxicity from long exposure and much washing by rain the conidia take the stain in varying degrees. Those that have become plasmolyzed become deeply stained, whereas those that have not become plasmolyzed, but the germination of which has been inhibited, appear unstained. Tests performed with residues known to contain no copper did not show the color when treated with chromotropic solution, whether or not the conidia had been killed.

The development and fixation of the purple color demonstrates the actual intake of copper by living cells. It is interesting that the copper fixed within the cells appeared to have the same power to react with the chromotropic solution as that in the solution surrounding the cells. From this it would seem that the copper may be fixed rather loosely in the plasma of the cell. On the other hand, conidia which have absorbed the copper during their growth activities have been subjected to lengthy periods of washing without lessening the density of the color inside the cells. Conidia as well as mycelium

killed prior to their contact with soluble copper readily absorb this material; but the copper is removed by washing. The copper is firmly fixed, once it is absorbed by living cells.

The data presented in table 2 are from one of a series of tests made as described. Data on exposure, rainfall, and conidial germination were obtained as for table 1. At the end of the incubation period conidia were transferred from each of the treated cover slips to potato agar. Chromotropic solution was then added to each slip and observations were made of the presence of copper within the cells, in the suspension liquid, and on the particles of the spray residue.

As indicated in table 2, scattered development of very short germ tubes, or buds,<sup>4</sup> was noticed toward the end of the tests with bordeaux mixture, but the conidia are shown to have been killed notwithstanding. The cell contents of all gave positive reactions for copper. These residues also gave indications of considerable copper by the nitric acid test. The parallel tests of zinc-lime were included to show the absence of effect of the excess lime as mentioned on page 521. Zinc-lime is toxic to these conidia for a day or so, and it is believed that the end of this period of toxicity indicates the change of alkalinity due to the change of lime in the residue to calcium carbonate. With zinc-lime residues no indications of the presence of copper were obtained in the conidia, in the solution, or on the spray particles.

TABLE 2.—*Presence of copper inside the conidia of Sclerotinia fructicola and its effect on their subsequent growth after a 24-hour incubation period*

Residue	Days weathered	Rainfall (total)	Germination	Subsequent growth on potato agar	Fixation of copper in cell contents <sup>1</sup>
		<i>Inches</i>	<i>Percent</i>		
Zinc-lime 4-4-50.....	1	0.00	0	Excellent.....	None.
	5	.02	85	.....do.....	Do.
	8	.02	97	.....do.....	Do.
	12	.17	15	.....do.....	Do.
	15	.67	98	.....do.....	Do.
	21	1.16	96	.....do.....	Do.
Bordeaux 2-4-50.....	1	.00	0	None.....	Marked.
	5	.02	0	.....do.....	Do.
	8	.02	(?)	.....do.....	Do.
	12	.17	(?)	.....do.....	Do.
	15	.67	(?)	.....do.....	Do.
	21	1.16	(?)	.....do.....	Do.

<sup>1</sup> Based on depth of purple color in test spores following treatment with chromotropic solution.

<sup>2</sup> Few buds (primordial germ tubes).

The data in table 2 demonstrate the ability of the bordeaux residues to resist weathering and to furnish a continual supply of available copper. Not a great deal of precipitation occurred during the long period of exposure, and although at times slight evidence of germination was noted, the amount of available copper in the precipitates was not greatly diminished at any time during the test; this probably accounts for the greater toxicity of these residues as compared with those cited in table 1.

<sup>4</sup> The term "bud" refers to the primordial germ tube, or beginning of the germ tube.

## EFFECT OF CELL ACTIVITY

In the work presented in table 1 it was noticed that after the residues had been subjected to a certain amount of weathering the conidia of a test suspension were not all affected alike. The escape of a few when nearly all were killed and the killing of a few when nearly all escaped suggested that some conidia had taken up copper more readily than others, and the color produced in them by the chromotropic solution confirmed this. To find the cause of this variation the work presented in table 3 was undertaken.

TABLE 3.—*Effect of cell activity on the absorption of copper by the conidia of Sclerotinia fructicola*

Incuba- tion period (hours)	Germina- tion of checks	Subsequent growth of checks	Germina- tion of treated conidia	Treated cells ab- sorbing copper <sup>1</sup>	Subsequent growth of treated conidia
	Percent		Percent	Percent	
0 0	0	Excellent.....			
1 0	7	-----do-----	0	13	Excellent.
2 0	25	-----do-----	0	41	Do
3 0	41	-----do-----	0	65	Do.
4 5	74	-----do-----	0	72	None.
23 5	80	-----do-----	0	80	Do.

<sup>1</sup> Based upon the development of a purple color inside the test spores following treatment with chromotropic solution

Two parallel series of germination tests were made. The cover slips in one series were without spray residue and were held as checks, whereas those in the other series were sprayed with bordeaux mixture and dried. All were started together, and at definite intervals one cover slip of each series was examined for germination. After the percentage of germinated conidia had been recorded, some of the conidia were transferred from the check and the treated cover slips to potato agar. The remainder of the conidia in contact with the spray residue were treated with chromotropic solution and examined under the microscope for evidence of copper absorption (table 3).

It was observed that conidia taken from the sprayed slips near the end of the test did not show any subsequent growth, whereas those transferred earlier did. From the data on germination of the check conidia (column 2 of table 3) it is apparent that the number of conidia that germinated in the early part of the test corresponded roughly with the number of spores that absorbed copper (column 5). It appears from this that those conidia that did not become active had not absorbed copper, since the chromotropic solution did not stain their protoplasm, but they were capable of germination and growth when transferred to a suitable medium.

To obtain further information on the relation of absorption to toxicity and to be sure that lack of absorption was not caused by a lack of soluble copper in contact with the conidia, the following experiments were conducted. Conidia from a string bean culture 8 days old were suspended in undiluted prune juice. This suspension was placed in the bottom of a beaker as a thin film so that germination or activity could proceed with an abundant supply of oxygen at room temperature.

At intervals drops of this suspension, which was of such density that about 50 conidia occupied a low-power objective field, were transferred to glass slides and an equal amount of a 2-percent copper sulphate solution was added. Thus the final concentration of copper sulphate was 1 percent. After an absorption period of 30 minutes the conidia were washed free of the copper salt and nutrient medium and then treated with the chromotropic solution. Records were then made of the conidia colored by the chromotropic solution and of germination or lack of germination, and finally some of the treated cells were transferred to potato agar. The results, as indicated in table 4, clearly show that although sufficient copper ions were present to cause toxicity to all the conidia, nevertheless in the early stages of germination many of the conidia did not absorb the copper ions. Absorption was correlated with germination activity, as is seen by comparing the percentage of conidia showing the color reaction and the percentage of germination in the check at the end of the same incubation periods. The observations clearly demonstrate that only those cells that become very active absorb the soluble copper. This was again apparent when some of the cells that had pushed out their primordial germ tubes and others that were obviously just beginning this process became deeply stained, while all the others, still dormant, remained unstained. As soon as all the conidia had become colored no subsequent growth appeared in any of the transfers to nutrient potato agar. One cannot escape the conclusion that the coloration is an indication of the absorption of a lethal dose of copper.

TABLE 4.—*Absorption of copper ions, from a 1-percent solution of copper sulphate, by the conidia of Sclerotinia fructicola*

Time of test <sup>1</sup>	Conidia	Check conidia germinating	Treated conidia colored <sup>2</sup>	Subsequent growth of treated conidia	Subsequent growth of check conidia
	Number	Number	Number		
Sept. 28 1934					
11 a. m. ....	69	0	0	Excellent.....	Excellent
12 m. ....	76	12	18	do.....	Do
1 p. m. ....	53	35	41	do.....	Do.
2 p. m. ....	62	62	62	None.....	Do.
3 p. m. ....	75	71	73	do.....	Do.
4 p. m. ....	70	68	70	do.....	Do
Sept. 29:					
9 20 a. m. ....	62	0	0	Excellent.....	Do.
9 30 a. m. ....	56	0	10	do.....	Do.
9 50 a. m. ....	62	0	14	do.....	Do.
10 a. m. ....	75	6	21	do.....	Do.
10 20 a. m. ....	69	18	31	do.....	Do.
10 40 a. m. ....	71	21	36	do.....	Do.

<sup>1</sup> 2 separate tests.

<sup>2</sup> Colored by the reaction of copper with chromotropic solution.

It is also apparent from the combined results of these experiments that not all the conidia began germination at the same time. In residues of bordeaux mixture, where the supply of soluble copper may become temporarily depleted, those conidia that start germination first may absorb a lethal quantity of the available copper, while those that start later may find the supply of this copper lowered to a point where only inhibition results. It is even possible that some conidia may escape entirely during this temporary depletion. It appears

from this that the available copper of bordeaux mixture is not readily or quickly replaced. These factors, therefore, may be responsible for the inhibitory but nonlethal effects of potentially fungicidal copper residues so frequently encountered under field conditions. As long as a sufficient residue is present, these periods of depletion of available copper must in the main be only transitory, since it has been found that a supply of available copper will begin to accumulate as soon as those factors causing removal of the available copper have ceased to operate.

#### BEHAVIOR OF GERM TUBES IN CONTACT WITH BORDEAUX MIXTURE RESIDUES

The procedure just described was also applied to conidia that had been incubated in a favorable medium until germ tubes had formed. In this way it was sought to compare the susceptibility of the germ tubes to the action of the spray residues with that of the conidia before and during germination, and again to demonstrate the intake of copper by means of the chromotropic solution.

In this part of the study two types of experiments were conducted. In both sets the procedure was essentially the same, in that the conidia were germinated in dilute prune juice before their transfer to the bordeaux mixture residues.

The residues used in both of these experiments were not weathered in the orchard environment, but had been prepared and stored in the laboratory fully a month before their use. The excess lime at that time had become fully carbonated, as was demonstrated by tests with phenolphthalein. The residues used carried a good supply of available copper, as indicated by the reaction with chromotropic solution.

In the first set of experiments the conidia were transferred directly without freeing them of any adhering metabolic and nutrient products, while in the second set the germinated conidia with their germ tubes were washed for 24 hours in tap water to free them from these materials insofar as practicable before their transfer to the bordeaux mixture residues.

In the first set of experiments, representative germ tubes were measured at the time of the transfers and again after certain intervals, depending upon the experiment. Some of the treated germinated conidia were tested with chromotropic solution and some were transferred to a suitable culture medium. In this manner observations were conducted on absorption of copper by the germ tubes and its effect on their subsequent growth. The results of such an experiment are presented in table 5.

The measurements recorded show that while the transfer had little effect upon the growth of the control germ tubes, the growth of the others was stopped almost instantly when they came in contact with the bordeaux mixture residues. Their failure to grow when transferred to a suitable medium indicated that the copper absorbed was lethal.

The conidia and germ tubes that had been in contact with the residues showed a slight plasmolysis of the protoplasm and became colored a deep purple upon treatment with chromotropic solution. The distribution of the copper, as indicated by the purple color, was found to be uniform throughout the entire protoplasmic system. The cell walls appeared not to absorb or fix the copper.



TABLE 5.—*Effect of absorption of copper by the germ tubes of conidia of Sclerotinia fructicola on the length growth of the tubes and on subsequent activity*

Time of measurement <sup>1</sup>	Check germ tubes		Treated germ tubes		
	Length growth	Subsequent growth	Length growth	Absorption of copper <sup>2</sup>	Subsequent growth <sup>3</sup>
11 a. m. ....	129	Excellent.	108	Some faintly colored.....	Excellent.
1 p. m. ....	138	...do....	93	Some well colored but most faintly colored	None.
2 p. m. ....	201	...do....	86	Most well colored, some faintly.	Do.
3 30 p. m. ....	194	...do....	108	Most well colored.....	Do.
10.30 a. m. (following day)...	720	...do....	108	All well colored.....	Do.

<sup>1</sup> Germinated conidia were transferred at 10 a. m.

<sup>2</sup> Based upon the depth of the purple color following treatment with chromotropic solution.

<sup>3</sup> Subsequent growth determined by transferring some of the check and some of the treated conidia, with their germ tubes, to potato hard agar slants

In the second set of experiments the germinated conidia, with their attached germ tubes, as already stated, were washed for 24 hours before being transferred to the clean or to the residue-covered slips. This procedure was designed to prevent, as far as possible, the staling products and nutritional materials from either increasing or lessening the quantity of available copper in the bordeaux-mixture residues. With such a treatment a small quantity of carbon dioxide might accumulate in the vicinity of each conidium, but it appears hardly likely that this amount could have any great influence.

The germinated conidia were transferred from the washed suspension to the clean or to the residue-covered slips. After intervals of ½ hour, 1 hour, 4 hours, and 24 hours, some of the check and some of the treated organisms were transferred from the slips to potato hard agar to determine the toxic effects of the copper. At the same time the remainder were treated with chromotropic solution and examined for copper within the cells.

The results showed that the available copper was absorbed by the germ tubes and conidia in lethal quantities during every period of contact with the residue. Thus the effect was even more pronounced than when the staling and nutritional products were not removed. The excellent growth of all the untreated transfers showed that the washing treatment was in no way deleterious.

In the section just preceding it was shown that the absorption of copper by conidia took place at so early a stage of activity that no evidence of initial germination could be seen under the microscope, either in conidia that had already absorbed an injurious quantity of copper or in the controls that had been incubated for the same length of time. In the work just described it is shown that when conidia that are already germinating are placed in contact with a source of available copper they too are immediately killed, as is evidenced by the instant cessation of tube growth.

## DISCUSSION

From the work reported in this paper it is evident that the residue is altered by exposure to weathering, since it then behaves somewhat differently from freshly formed residue. The work presented here

also shows that when this change has gone so far that sufficient quantities of available copper are not present the application of additional sprays to maintain continuous protection is required. The changed behavior indicates that some form of copper present in the beginning is not present at the end of prolonged weathering and that the forms of copper that remain are not able to maintain the original high concentration in the solution in contact with them.

The proportion of highly soluble copper in the fresh deposit and the rapidity with which it can be exhausted by washing remain to be determined. From this work it appears that the amount is not large and that the appearance of the residue does not give warning of its depletion. As weathering progresses, an increasing proportion of the conidia seeded upon the residue retain their viability, although germination is prevented.

It must be emphasized, therefore, that the bordeaux mixture residues which are commonly applied to plants and which have been found to be injurious to many of them, according to the environmental conditions encountered, are toxic to many of the fungus spores which come in contact with them from the moment they are applied. These experiments have clearly shown the reasons for this, and it appears certain that the vagrant fungus conidia that fall on these residues come in contact with sufficient available copper to cause toxicity, provided the residues are not unduly washed or depleted.

Other evidence in regard to the availability or nonavailability of the copper of spray residues is found in the results of tests with the chromotropic solution. The direct application of this reagent gives a color the depth of which is roughly proportional to the toxicity as determined in the usual way.

The adherence of most of the residue is shown to be quite satisfactory. Although after 5 days the seeded spores showed themselves able to germinate after transfer (table 1), the residues continued to prevent germination during 37 days in spite of the number of heavy rains. In table 2, on the other hand, no growth was recorded after transfer on bordeaux mixture residues weathered for 21 days. This is attributed to the fact that decidedly less rain fell during the course of this experiment, and very little during the first 12 days (table 2). It also suggests that the foliage injury which often follows long periods of fog, heavy dew, or showers may be caused by the maintenance of a copper solution upon the leaves.

It has been observed repeatedly that the absorption of copper takes place only in cells that become active and not in those that are dormant or inactive. This has been very well shown in the experiments. The results obtained with cells washed relatively free of their metabolic products clearly demonstrate that such cells are capable of absorbing directly from bordeaux mixture residues a form of copper causing a lethal effect. These copper materials, when in rather low concentrations, were found to be only inhibitory in effect.

The mechanism of the lethal effect has not been clearly worked out. It is certain that the copper becomes dispersed throughout the protoplasmic mass, for the color produced when the loosely combined soluble copper reacts with the chromotropic molecule is evenly distributed. These experiments show that inactivation follows the absorption of the copper and that the activity of the cell menstruum is responsible for the intake of the copper ions. It has also been

observed that the reaction of the copper ions with the cell menstrium is a permanent one, for after the death of the cells no amount of washing frees the injured cells of the copper that has been absorbed. Nevertheless the copper is not so firmly bound in its combination with the elements of the cytoplasm as to prevent its usual response to treatment with the chromotropic solution.

It has been the constant experience of the writers that at times the amount of copper in solution is only of such magnitude as to cause inhibition. The study of inhibition is an interesting one, since it must take into consideration an entirely different set of conditions from those that obtain with the active cell. It is difficult to understand the phenomenon of inhibition when the theory of toxicity is applied to it, except as something different in kind as well as in degree from toxicity.

While these studies offer a satisfactory explanation of the effect of the available copper of bordeaux mixture residues on sensitive organisms, they do not attempt to define those systems concerned with organisms apparently resistant to copper. Such systems require special study.

#### SUMMARY AND CONCLUSIONS

A study is presented in this paper of the effect on the conidia of *Sclerotinia fructicola* (Wint.) Rehm of the available copper inherent in the residues of bordeaux mixture that have been exposed to weathering and aging.

This study also includes the effect on germinated conidia of *Sclerotinia fructicola* of the available copper inherent in the residues of bordeaux mixture that have been aged in the laboratory.

A new procedure, namely, exposure of the residues to the environment of the host plants, has been added to the usual methods of estimating the effect of fungicides. This method furnishes a means of weathering residues of fungicides under the same environmental conditions as those which affect the spray residues on the host plants.

As a further precaution in the proper evaluation of the effects of fungicides on fungus spores, some of the test spores were transferred to a suitable nutrient medium in order to determine whether the effect of the test materials was toxic or merely inhibitory.

It has been determined, confirming the work of others, that the soluble copper inherent in the precipitates of the residues of bordeaux mixture is in the proportion of at least four parts per million by volume of the original mixture.

The soluble copper in residues, in solution, and in the protoplasm of affected conidia has been detected by the use of a very sensitive reagent, namely, the sodium salt of nitroso-chromotropic acid, which is known as the "chromotropic solution."

It has been demonstrated that the copper of the residues of bordeaux mixture is absorbed by the conidia of *Sclerotinia fructicola*, causing death or inhibition according to the amount of copper available.

The absorption of the copper by the conidia from the residues of bordeaux mixture is correlated with the initiation of activity, and cells that remain dormant or are delayed in their activity do not absorb copper.

Conidia of *Sclerotinia fructicola* have been found to be inhibited in their growth when the supply of copper has not been quite sufficient

to cause toxicity. These inhibited conidia readily become active when transferred to a suitable culture medium.

It is indicated that where the quantity of soluble copper in a depleted residue is small, it may be exhausted by absorption into the conidia that begin their activity first, and that conidia that become active later escape the injurious effect.

When actively growing conidia were transferred to an aged bordeaux mixture residue, but one in which the inherent soluble copper had not been depleted, both the conidial and attached germ-tube cells absorbed the copper with lethal effects.

The absorption of the lethal dose of soluble copper from aged residues by the germ tubes of the conidia of *Sclerotinia fruticola* was found to cause an immediate cessation of germ-tube elongation.

The soluble copper that is absorbed by the conidia and germ tubes has been found to be distributed evenly throughout the protoplasmic mass, which stains purple when treated with chromotropic solution.

The color that is formed by the combination of chromotropic solution with the copper absorbed in the cells has been found to be similar to that which simple copper ions form with chromotropic solution in vitro.

It is concluded that in freshly deposited bordeaux mixture residues there is present a component bearing available copper which is exhausted by washing with rains but not by aging. After it is lost, conidia that come to rest in contact with the residue are inhibited in germination but are not killed, and can germinate freely when removed from this contact. While it is still present, conidia take up copper before any visible evidence of vital activity is observed. From this it appears that the conidial cells are not capable of secreting a substance which will dissolve the inert copper of these washed residues.

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# INHERITANCE OF CHLAMYDOSPORE CHARACTERISTICS IN OAT SMUT FUNGI<sup>1</sup>

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## INTRODUCTION

The nature of inheritance in the smut fungi has been the subject of rather extensive investigations in recent years. Primary emphasis in these investigations has been placed on a study of the segregation of factors for sex and cultural characters, whereas little has been done towards determining the nature of inheritance of chlamydospore characteristics. In previous reports<sup>3</sup> the writer has presented preliminary evidence to show that certain chlamydospore characteristics in the oat smut fungi are inherited according to the Mendelian law of segregation and recombination. Results are now available from more extensive investigations and are here reported.

## MATERIAL AND METHODS

Loose smut of oats, *Ustilago avenae* (Pers.) Jens., covered smut of oats, *U. levis* (Kell. and Sw.) Magn., and the "buff smut" fungus<sup>4</sup> were used in these studies. They hybridize readily, and their respective chlamydospore characteristics are morphologically distinct. *U. avenae* has echinulate brown chlamydospores; *U. levis* has smooth brown chlamydospores; and the buff smut fungus has smooth hyaline chlamydospores. In hybrids between these species, therefore, it is possible to study the nature of segregation and recombination of factors for chlamydospore markings and color both in single and in double factor pairs.

Hybrid material was obtained by inoculating 25 oat seedlings (*Avena sativa* L.) with crosses between monosporidial lines of *Ustilago levis* and the buff smut fungus, between *U. levis* and *U. avenae*, and between *U. avenae* and the buff smut fungus. The ratios in the F<sub>2</sub> segregations were determined on the basis of types of individuals (chlamydospores) produced by inoculating with F<sub>1</sub> chlamydospores or with crosses between F<sub>1</sub> monosporidial lines. The observed distribution of the F<sub>2</sub> populations of all hybrids was compared with the expected distribution and tested by Fisher's formula for testing goodness of fit,  $\chi^2 = S\left(\frac{\chi^2}{m}\right)$ .<sup>5</sup>

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## EXPERIMENTAL DATA

USTILAGO LEVIS  $\times$  BUFF SMUT

In hybrids between *Ustilago levis* (chlamydospores brown) and the buff smut fungus (chlamydospores hyaline) the  $F_1$  chlamydospores were brown, indicating dominance of the factor for brown. In these hybrids approximately 50 percent of the  $F_1$  sporidia were viable. Two or more monosporidial lines were obtained from each of 8  $F_1$  chlamydospores, giving a total of 22, and these  $F_1$  monosporidial lines were crossed in all sexually compatible combinations to obtain the  $F_2$  generation.

As indicated in table 1, the  $F_2$  population was distributed into two classes. In four of the chlamydospores (45, 47, 49, and 57) segregation for sex occurred in the first division while in the other four (50, 53, 54, and 55) segregation for sex occurred in the second division of the diploid nucleus. In all the  $F_1$  chlamydospores, except 49 and 54, segregation for color occurred in the second division of the diploid nucleus. In chlamydospores 45, 47, 54, and 57 segregation for sex and color occurred in separate nuclear divisions, while in chlamydospores 50, 53, and 55 segregation for sex and color occurred in the same (second) division of the diploid nucleus. It is therefore evident (table 1) that the  $F_2$  population of these seven chlamydospores was distributed into two classes in a 3:1 ratio. In chlamydospore 49, however, segregation for sex and color occurred in the first division of the diploid nucleus, and consequently the  $F_2$  population of this spore was brown. Nevertheless, segregation for color and sex occurred in the  $F_2$  of this spore in such a way as to produce brown and hyaline spores in the  $F_3$ . On the basis of these data it is apparent that the  $F_2$  distribution of color in *Ustilago levis*  $\times$  buff smut hybrids is in a simple 3:1 ratio.

TABLE 1.—Segregation of factors for color<sup>1</sup> and sex in eight chlamydospores of a *Ustilago levis*  $\times$  buff smut hybrid

F <sub>1</sub>						F <sub>2</sub>					
Chlamydo- spore no. <sup>2</sup> and genotype	Spori- dium no.	Sex <sup>3</sup>	Gam- etes	Geno- type	Ratio	Chlamydo- spore no. <sup>2</sup> and genotype	Spori- dium no.	Sex <sup>3</sup>	Gam- etes	Geno- type	Ratio
5 (Bb)-----	1	+	b	BB	1:2:1	50 (Bb)-----	1	+	B	BB	1:2:1
	2	+	b	Bb			2	+	b	Bb	
	3	—	b	Bb			3	+	b	Bb	
	4	—	B	bb			4	—	B	bb	
47 (Bb)-----	1	+	B	BB	1:2:1	53 (Bb)-----	1	+	b	BB	1:2:1
	2	+	b	Bb			2	—	B	Bb	
	3	—	B	Bb			3	—	b	Bb	
	4	—	b	bb			4	+	B	bb	
49 (Bb)-----	1	+	b	Bb	4:0	54 (Bb)-----	1	+	B	BB	1:2:1
	2	+	b	Bb			2	—	B	Bb	
	3	—	B	Bb			3	+	b	Bb	
	4	—	B	bb			4	—	b	bb	
57 (Bb)-----	1	+	b	BB	1:2:1	55 (Bb)-----	1	+	b	BB	1:2:1
	2	+	B	Bb			2	—	B	Bb	
	3	—	b	Bb			3	—	b	Bb	
	4	—	B	bb			4	+	B	bb	

<sup>1</sup> The factor for brown, carried by *U. levis*, is represented by B, and the factor for hyalineness, carried by the buff smut, is represented by b.

<sup>2</sup> Numbers assigned to the chlamydospores from which single sporidia were isolated.

<sup>3</sup> Plus (+) and minus (—) signs indicate sporidia of opposite sex.



The advantage of using crosses between  $F_1$  monosporidial lines for determining the class distribution of the  $F_2$  population is obvious. This advantage is lost, however, unless two or more  $F_1$  sporidia can be obtained from each promycelium. This was possible only in the *Ustilago levis*  $\times$  buff smut hybrids. It therefore became necessary to use  $F_1$  chlamydospores of other hybrids for inoculum in order to obtain an  $F_2$  population. To test the dependability of this method of determining the  $F_2$  distribution, an  $F_2$  population of the *U. levis*  $\times$  buff smut hybrid, the  $F_2$  distribution of which had previously been determined by inoculating with crosses between  $F_1$  monosporidial lines, was obtained by inoculating with  $F_1$  chlamydospores. By using each smutted panicle as an individual<sup>6</sup> the class distribution was determined. The  $F_2$  population of 1,078 individuals (smutted panicles) was distributed into two classes, as shown in table 2.

TABLE 2.—Segregation for color in the  $F_2$  of the hybrid *Ustilago levis*  $\times$  buff smut

Color	$F_2$ individuals	
	Observed	Expected
Brown (chlamydospores brown).....	Number 826	Number 809
Buff (chlamydospores hyaline).....	252	269
Total.....	1,078	1,078

These data are in close agreement with the expected 3:1 ratio ( $P=0.24$ ) and serve to indicate the dependability of this method for determining the nature of segregation and recombination of factors for chlamydospore characteristics in oat smut hybrids.

USTILAGO AVENAE  $\times$  U. LEVIS

In hybrids between *Ustilago avenae* (chlamydospores echinulate) and *U. levis* (chlamydospores smooth) the  $F_1$  chlamydospores were echinulate, indicating dominance of the factor for echinulate spore walls over the factor for smooth spore walls. The  $F_2$  population was obtained by inoculating with  $F_1$  chlamydospores, and the 235 individuals (smutted panicles of the  $F_2$ ) were distributed into two classes, as shown in table 3.

TABLE 3.—Segregation for smoothness in the  $F_2$  of the hybrid *Ustilago avenae*  $\times$  *U. levis*

Character	$F_2$ individuals	
	Observed	Expected
Echinulate.....	Number 185	Number 176
Smooth.....	50	59
Total.....	235	235

<sup>6</sup> The panicle rather than the plant was used as the unit because both types were sometimes found on the same plant but in separate panicles.

These figures are adaptable to a theoretical ratio for the  $F_2$  of a cross involving a single-factor difference ( $P=0.18$ ). Apparently, then, the distribution of the  $F_2$  population into echinulate and smooth chlamydospore classes in *U. avenae*  $\times$  *U. levis* hybrids is on a simple 3:1 basis.

#### USTILAGO AVENAE $\times$ BUFF SMUT

In hybrids between *Ustilago avenae* and the buff smut fungus two character pairs are involved, viz, chlamydospore markings and color. Taken separately, as in *U. avenae*  $\times$  *U. levis* and *U. levis*  $\times$  buff smut hybrids, respectively, these character pairs give a 3:1 ratio. Therefore, in *U. avenae*  $\times$  buff smut hybrids it might be expected that the typical dihybrid ratio 9:3:3:1 would be obtained. This, however, is not the case. The  $F_2$  population, which was obtained by inoculating with  $F_1$  chlamydospores, was distributed into three of the four expected classes, as shown in table 4.

TABLE 4.—Segregation for color and smoothness in the  $F_2$  of the hybrid *Ustilago avenae*  $\times$  buff smut

Characters	$F_2$ individuals	
	Observed	Expected <sup>1</sup>
	Number	Number
Brown echinulate.....	499	490
Brown smooth.....	175	164
Hyaline echinulate.....	0	164
Hyaline smooth.....	199	55
Total brown.....	674	654
Total hyaline.....	199	219
Total echinulate.....	499	654
Total smooth.....	374	219
Total individuals.....	873	873

<sup>1</sup> On the basis of the 3 classes into which the  $F_2$  population was actually distributed, the expected numbers are as follows. Brown echinulate, 490, brown smooth, 164, hyaline smooth, 219.

If the double recessive class, hyaline smooth, had appeared in the expected proportion it might have been logical to attribute the absence of the hyaline echinulate class to lethal factors. However, there were almost four times as many smooth hyaline individuals as were expected, and the total number of hyaline individuals, as a class, was about equal to the number that was expected, on a 9:3:3:1 basis. In other words, considering the single-factor pair for color independently, the 873  $F_2$  individuals were distributed into two classes, brown and hyaline (table 4). The observed distribution is within the theoretical limits of a 3:1 ratio ( $P=0.16$ ). The fact that all of the hyaline individuals had smooth walls seems to indicate that the expression of echinulation in the hyaline chlamydospores is suppressed. Furthermore, considering the single-factor pair for chlamydospore markings, the 873  $F_2$  individuals were distributed into two classes, echinulate and smooth (table 4).

Obviously, this observed distribution does not agree with the expected distribution, on a 3:1 basis, because of the absence of the hyaline echinulate class ( $P<0.01$ ). However, on the assumption that expres-

sion of echinulation is suppressed in hyaline spores, the observed distribution of echinulate and smooth chlamydospores shown in table 4 is about what would have been expected.

If, on the other hand, hyaline echinulate spores had been produced in the proper proportion for a 9:3:3:1 ratio, then the  $F_2$  population would have been distributed into two classes, as shown in table 5.

TABLE 5.—Hypothetical distribution of  $F_2$  population of hybrid *Ustilago avenae*  $\times$  buff smut

Character	$F_2$ individuals	
	Assumed as observed	Expected
Echinulate.....	Number 663	Number 654
Smooth.....	210	219
Total.....	873	873

It is evident that the distribution assumed as observed of the  $F_2$  population into echinulate and smooth classes, based on the assumption that hyaline echinulate individuals did occur in the expected proportion for a 9:3:3:1 ratio, is agreeable with the expected distribution for a 3:1 ratio ( $P=0.48$ ). Apparently, then, there is reason to assume the presence of an inhibitor which prevents the expression of the factor for echinulation in the hyaline spores of the buff smut fungus. If this is true, it would be expected that the  $F_2$  population of *Ustilago avenae*  $\times$  buff smut hybrids would be distributed in proper proportions for a 9:3:4 ratio. The 873 individuals of this cross were distributed into three classes, brown echinulate, brown smooth, and hyaline smooth (table 4).

That the distribution of the  $F_2$  population of the *Ustilago avenae*  $\times$  buff smut hybrid is in agreement with the expected distribution for a 9:3:4 ratio ( $P=0.26$ ) seems to justify the assumption that an inhibitor is present which suppresses echinulation in the hyaline chlamydospores. If the inhibitor hypothesis is correct, then half of the hyaline gametes would carry the factor for echinulation and should produce echinulate brown  $F_1$  chlamydospores when crossed with *U. levis*. Crosses of this type, which would be final proof for the inhibitor hypothesis, have not as yet been made.

#### SUMMARY

The factor for brown is dominant over the factor for hyaline chlamydospores in hybrids between covered smut of oats, *Ustilago levis* (chlamydospores brown, smooth), and the buff smut fungus (chlamydospores hyaline, smooth), and the distribution of the  $F_2$  population is on a simple 3:1 basis.

The factor for echinulate spore walls is dominant over the factor for smooth spore walls in hybrids between loose smut of oats, *Ustilago avenae* (chlamydospores echinulate), and covered smut of oats, *U. levis* (chlamydospores smooth), and the  $F_2$  population of echinulate and smooth chlamydospores was distributed on a 3:1 basis.

In hybrids between *Ustilago avenae* (chlamydospores brown, echinulate) and the buff smut fungus (chlamydospores hyaline, smooth) factors for echinulation and brown are dominant over factors for smoothness and hyalineness, respectively. The  $F_2$  population was distributed into echinulate brown, smooth brown, and smooth hyaline classes in a 9:3:4 ratio. The evidence seems to indicate that the presence of an inhibitor prevents the expression of echinulation in the hyaline chlamydospores of the buff smut fungus.

# PENETRATION OF TRICHODERMA LIGNORUM INTO SAPWOOD OF PINUS TAEDA<sup>1</sup>

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## INTRODUCTION

*Trichoderma lignorum* (Tode) Harz is the most common mold found growing on the unseasoned sapwood of pine. During warm, humid weather and under improper methods of seasoning, the sapwood surfaces of some species may be partially covered by this fungus within a few days after sawing. In general, lumbermen disregard the presence of this fungus because they consider it a superficial growth causing only a surface discoloration which disappears after the lumber is seasoned. So far as the writer knows no experimental data have been published which show that *Trichoderma lignorum* penetrates wood. Gerry,<sup>3</sup> however, showed that five other wood-inhabiting molds penetrated both hardwoods and softwoods. Falck<sup>4</sup> stated that water-stored wood is often attacked to a certain degree by the so-called green "Holzschimmel" (*Trichoderma lignorum*). He also stated that this mold may spread very rapidly over the outer surface and to the interior of the wet wood without influencing the strength of the wood.

*Trichoderma* has frequently been isolated by the writer from the interior of wood, indicating that it is more than a superficial growth on the wood. The following experiments were therefore undertaken to determine the extent and rapidity at which *Trichoderma lignorum* would grow through the sapwood of loblolly pine (*Pinus taeda* L.). The first results obtained were sufficient to prove that the fungus penetrates steam-sterilized wood rapidly. Subsequent investigations were made to determine the effect of steam sterilization on the susceptibility of the wood to the invasion of the fungus, and the part played by the moisture content of the wood.

## EXPERIMENTAL DATA

### METHODS

Five strains of *Trichoderma lignorum* were isolated by the spore-dilution method from southern yellow pine chips collected in mill yards in Mississippi and Louisiana. The cellulose-dissolving ability of these strains was tested on McBeth and Scales' cellulose agar.<sup>5</sup> The strain that cleared the agar most rapidly (no. 5) and the strain that

<sup>1</sup> Received for publication Dec. 2, 1935; issued April 1936. In cooperation with the Forest Products Laboratory, Forest Service, U. S. Department of Agriculture.

<sup>2</sup> The writer wishes to express her appreciation to C. A. Richards, Carl Hartley, R. M. Lindgren, and other members of the Division of Forest Pathology for helpful suggestions received during the investigation reported in this paper.

<sup>3</sup> GERRY, E. FIVE MOLDS AND THEIR PENETRATION INTO WOOD. Jour. Agr. Research 26: 219-229, illus. 1923.

<sup>4</sup> FALCK, R. UBER DEN EINFLUSS DES FLOSSENS AUF DIE WIDERSTANDSFÄHIGKEIT DES RATHOLZES GEGEN TROCKENFAULE UND ÜBER DEN HOLZSCHUTZ DURCH SCHIMMELBEFALL UND DIFFUSIONSTRÄNKUNG. Mitt. Forst u. Forstwiss. 11: 480-485, illus. 1931.

<sup>5</sup> MCBETH, I. G. and SCALES, F. M. THE DESTRUCTION OF CELLULOSE BY BACTERIA AND FILAMENTOUS FUNGI. U. S. Dept. Agr., Bur. Plant Indus. Bull. 266: 27-28. 1913.

did not clear it within 10 days after inoculation (no. 3) were selected for use in the penetration studies.

Experimental blocks, 2 by 2 by 2 inches, were quarter-sawed from the sapwood of the *Pinus taeda* logs collected in Louisiana and Florida. The wood was free from infection, the rate of growth was medium (from 4½ to 6 annual rings per inch), and the grain was fairly straight. The ends of the freshly cut logs had been treated with ethyl mercuric chloride and painted with filled hardened gloss oil to prevent invasion of fungi and loss of moisture.

Blocks in one group were selected at random and steamed for 30 minutes at 100° C. (atmospheric pressure). Blocks in another group

were selected so that those steamed for 30 minutes at 121° (15 pounds pressure) could be compared with longitudinally and tangentially matched unsteamed blocks. In a third group, blocks were steamed 30 minutes at 100° and compared with others that had been steamed 30 minutes at 121° and also with unsteamed blocks. Figure 1 shows how the blocks were selected.

To prevent *Trichoderma* from entering any side of the block except the one upon which the inoculation was to be made, all sides except that one were dipped in a hot bath of paraffin (about 100° C.) and

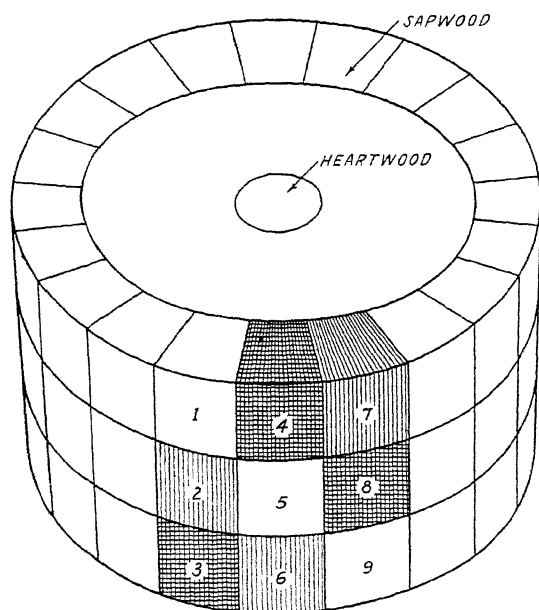


FIGURE 1.—Diagram showing how 2-inch cubes were cut from the log. Not shaded (1, 5, 9), not steamed; lightly shaded (2, 6, 7), steamed 30 minutes at 100° C.; heavily shaded (3, 4, 8), steamed 30 minutes at 121°.

held for 15 seconds, then immediately transferred to a cooler paraffin bath (about 60°) for 30 seconds. The object of the first paraffin bath was to penetrate the surface of the block sufficiently to hold the thicker paraffin film formed in the second bath.

The blocks were placed in sterile glass jars, 5 by 5 by 10 inches, containing 35 cc of distilled water. They were prevented from coming into direct contact with the water by means of glass supports. A layer of cotton was placed between the glass stopper and the rim of the jar.

As soon as the steamed blocks were cool they were inoculated with approximately 0.2 cc of a heavy spore suspension of *Trichoderma lignorum* placed on the center of the unparaffined side of each block. They were then incubated at from 25° to 30° C. for different periods.

At the end of the incubation period small cubes which were to be sectioned for microscopical examination were sawed from the inocu-

lated blocks (fig. 2). These cubes were boiled 15 minutes in distilled water and preserved in a 50-50 glycerin-alcohol mixture. From 10 to 20 radial sections  $30\mu$  thick were then taken from the cubes and examined under the microscope for the presence and depth of penetration of *Trichoderma* mycelium. The sections were stained 15 minutes in Pianeze III b,<sup>6</sup> dehydrated with alcohol, cleared with a solution of carbol-turpentine, treated with xylol, and mounted in Canada balsam.

Moisture determinations<sup>7</sup> of the wood were made at the end of the incubation period. The moisture samples consisted of a tangential and a radial side which remained after the center was removed from the 2-inch cube (fig. 2).

# RESULTS

The rate and extent of penetration of *Trichoderma lignorum*, strains nos. 3 and 5, into *Pinus taeda* sapwood that had been steamed 30 minutes at  $100^{\circ}$  C. are recorded in table 1. At the end of 72 hours both strains of the fungus had penetrated a 2-inch block which had been inoculated on the tangential side. At the end of 60 hours, strain no. 5 had penetrated a block inoculated on the transverse side, while strain no. 3 had penetrated only three-fourths of that distance.

The mycelium was present in the ray parenchyma cells, ray tracheids, wood tracheids, and resin ducts. The hyphae were most abundant in the ray parenchyma and wood tracheids (fig. 3). This was true for both strains. No evidence of direct penetration was seen. The mycelium appeared to pass through the natural openings.

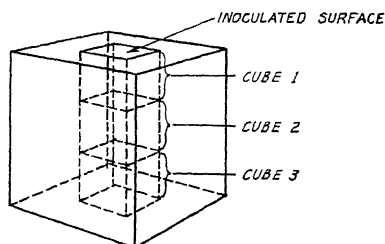


FIGURE 2.—Method of cutting blocks inoculated with *Trichoderma* in preparation for sectioning.

TABLE 1.—Rate of penetration of *Trichoderma lignorum* into *Pinus taeda* sapwood steamed 30 minutes at  $100^{\circ}$  C.

Incubation period (hours)	Strain no.	Distance penetrated		Incubation period (hours)	Strain no.	Distance penetrated	
		Radially	Longitudinally			Radially	Longitudinally
		<i>Inches</i>	<i>Inches</i>			<i>Inches</i>	<i>Inches</i>
24-----	{ 3	(1)	0.00	60-----	{ 3	0.88	1.50
	{ 5	(1)	0.00		{ 5	0.88	2.00
48-----	{ 3	0.19	1.06	72-----	{ 3	2.00	-----
	{ 5	0.06	0.63		{ 5	2.00	-----

<sup>1</sup> Less than 0.06 inch

The possibility that the rapidity of penetration was due to a change in the wood brought about by steaming was investigated. Longitudinally and tangentially matched steamed and unsteamed blocks

<sup>6</sup> VAUGHAN, R. E. A METHOD FOR THE DIFFERENTIAL STAINING OF FUNGUS AND HOST CELLS. Ann. Mo. Bot. Gard. 1: 241-242. 1914.

<sup>7</sup> Expressed as  $\left( \frac{\text{Original weight} - \text{oven-dry weight}}{\text{Oven-dry weight}} \right) 100$ .

were inoculated with *Trichoderma lignorum*, strain no. 5. The results (tables 2 and 3) show that steaming wood does affect the rate of penetration by *Trichoderma*. The average distance penetrated longitudinally by *Trichoderma* in 20 steamed 2-inch cubes in 60 hours was 1.71 inches (table 2), while the average distance penetrated in 20 matched unsteamed cubes was 0.45 inch.

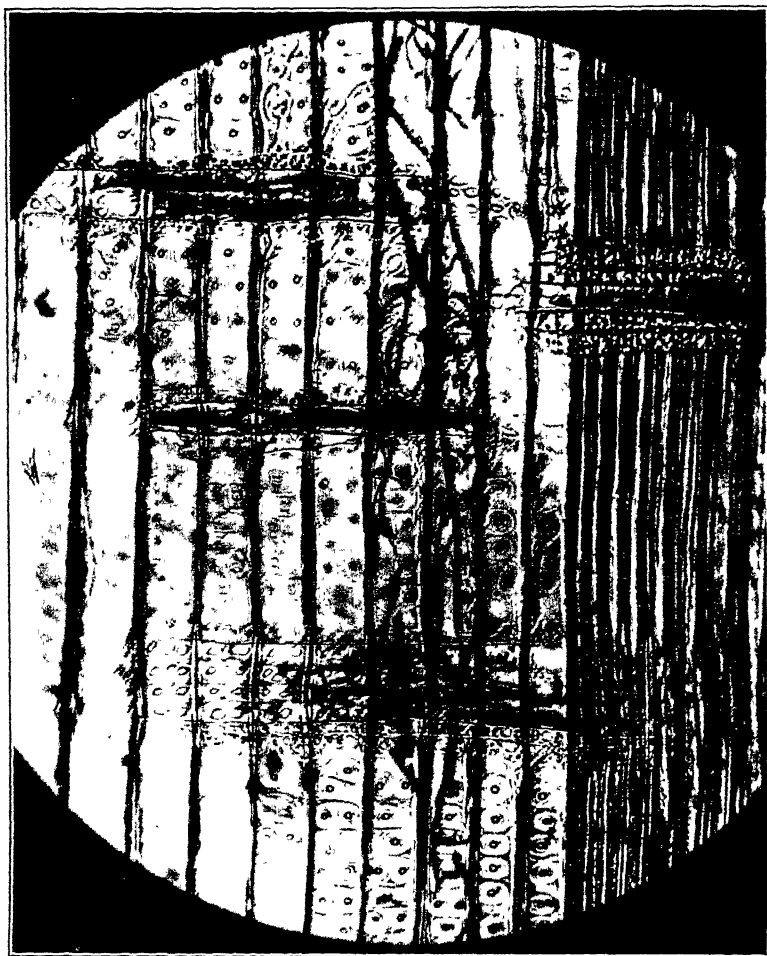


FIGURE 3—Photomicrograph showing *Trichoderma* mycelium in wood tracheids and ray parenchyma cells of *Pinus taeda* sapwood  $\times 165$ .

The relative effect of steaming for 30 minutes at 100° or 121° C. may be seen in table 3. The results indicate that the difference in temperature caused no significant difference in the rate of penetration by *Trichoderma*. The fungus penetrated 1.71 inches into wood steamed at 100°, and 1.63 inches into wood steamed at 121°. During the same period the fungus penetrated only 0.4 inch into the unsteamed wood.



TABLE 2.—Effect of steaming (at 121° C. for 30 minutes) on the longitudinal penetration of *Trichoderma lignorum* into sapwood of *Pinus taeda* in 60 hours

Unsteamed wood			Steamed wood		
2-inch cube no	Moisture	Distance penetrated	2-inch cube no	Moisture	Distance penetrated
	Percent	Inch		Percent	Inches
1.....	80.8	0.50	2.....	68.1	1.75
4.....	97.6	.38	3.....	77.8	1.88
5.....	81.3	.44	6.....	63.7	1.88
8.....	84.1	.50	7.....	65.0	1.50
9.....	82.6	.50	10.....	64.6	1.75
12.....	83.4	.56	11.....	71.0	1.94
13.....	78.1	.44	14.....	67.4	1.69
16.....	94.2	.44	15.....	72.3	1.94
17.....	89.5	.56	18.....	77.8	2.00
20.....	95.8	.44	19.....	79.2	1.81
21.....	86.8	.50	22.....	67.0	1.88
24.....	77.0	.38	23.....	66.6	1.56
25.....	86.0	.31	26.....	79.2	1.50
28.....	90.6	.56	27.....	66.6	1.81
29.....	90.4	.50	30.....	108.2	1.75
32.....	112.6	.25	31.....	97.5	1.56
33.....	95.2	.44	34.....	77.4	1.75
36.....	98.9	.50	35.....	87.7	1.50
37.....	96.4	.44	38.....	78.2	1.31
40.....	106.2	.38	39.....	79.1	1.50
Average.....	90.4	.45	Average.....	75.3	1.71

TABLE 3.—Effect of steaming (at 100° and 121° C. for 30 minutes) on the longitudinal penetration of *Trichoderma lignorum* into sapwood of *Pinus taeda* in 60 hours

Unsteamed wood			Wood steamed at 100° C			Wood steamed at 121° C		
2-inch cube no	Moisture	Distance penetrated	2-inch cube no	Moisture	Distance penetrated	2-inch cube no	Moisture	Distance penetrated
	Percent	Inch		Percent	Inches		Percent	Inches
1.....	104.3	0.31	2.....	100.1	1.69	3.....	81.9	1.50
5.....	118.4	.31	6.....	101.5	1.88	4.....	93.2	1.38
9.....	117.3	.44	7.....	93.1	1.56	8.....	62.5	1.94
10.....	111.0	.44	11.....	101.8	1.75	12.....	94.9	1.75
14.....	108.9	.38	15.....	102.3	2.00	13.....	88.7	1.50
18.....	104.6	.44	16.....	93.7	1.50	17.....	88.0	1.94
19.....	107.6	.50	20.....	97.7	1.94	21.....	86.2	1.75
23.....	104.6	.38	24.....	89.2	1.56	22.....	83.4	1.38
27.....	92.4	.38	25.....	81.3	1.50	26.....	77.4	1.50
Average..	107.7	.40	Average..	95.6	1.71	Average..	87.4	1.63

Since wood loses moisture during steaming, it was thought that perhaps the steaming might have reduced the moisture of the wood so that the steamed blocks contained only the amount required for the optimum growth of *Trichoderma*, while the unsteamed blocks were too wet. However, the highest moisture content (table 2) of any one steamed block was 108.2 percent, and the lowest moisture content of any steamed block was 63.7 percent; yet the fungus traveled only 0.13 inch farther in the latter. A similar relation existed in the unsteamed blocks. *Trichoderma* grew 0.38 inch into an unsteamed block having a moisture content of 106.2 percent and the same distance into another having a moisture content of 77 percent. The fungus grew very differently on steamed and unsteamed blocks having approximately the same moisture content (table 4).

TABLE 4.—Depth of penetration of *Trichoderma lignorum* into steamed (for 30 minutes at 121° C.) and unsteamed blocks of *Pinus taeda* sapwood having approximately the same moisture content

Unsteamed blocks		Steamed blocks	
Moisture (percent)	Depth of penetration	Moisture (percent)	Depth of penetration
	<i>Inch</i>		<i>Inches</i>
108.9.....	0.38	108.2.....	1.75
97.6.....	.38	97.5.....	1.56
86.8.....	.50	86.2.....	1.75
78.1.....	.44	78.2.....	1.31
77.....	.38	77.4.....	1.63

<sup>1</sup> Average for 2 blocks

### SUMMARY

The penetration of *Trichoderma lignorum* into sapwood of *Pinus taeda* was determined by the presence of the fungus in the ray parenchyma cells, ray tracheids, wood tracheids, and resin ducts. The greatest number of hyphae were found in the ray parenchyma and wood tracheids. This was true for both strains used.

The hyphae passed through the natural openings of the wood.

The fungus penetrated the wood both radially and longitudinally, but there was evidence that it grew more rapidly in the longitudinal direction.

There was no significant difference in the rate of penetration between the cellulose-dissolving strain and the noncellulose-dissolving strain.

*Trichoderma lignorum* penetrated both steamed and unsteamed sapwood; however, steamed wood was penetrated about four times faster than unsteamed wood. Steam sterilization at 100° C. for 30 minutes had the same effect as steam sterilization at 121° for the same length of time, so far as could be determined by the growth of the fungus in the wood.

The difference in moisture content between steamed and unsteamed wood did not appear to be the cause of the difference in the rate of penetration by *Trichoderma lignorum*.

# RELATIVE RESISTANCE TO BACTERIAL WILT OF CERTAIN COMMERCIAL AND SELECTED LOTS OF ALFALFA<sup>1</sup>

By J. L. WEIMER, *senior pathologist, Division of Forage Crops and Diseases, Bureau of Plant Industry, United States Department of Agriculture*, and B. A. MADSON, *head of Division of Agronomy, California Agricultural Experiment Station*

## INTRODUCTION

The United States Department of Agriculture and certain State experiment stations have published a number of papers (1-13)<sup>2</sup> giving the results of research seeking an alfalfa (*Medicago sativa* L.) resistant to bacterial wilt (*Phytomonas insidiosa* (L. McC.) Bergey et al.). Westover (12) has pointed out that Ladak, Hardistan, Kaw, and the commercial Turkistan alfalfas have shown varying degrees of resistance. All of these alfalfas, however, are of the winter-hardy type, which, regardless of temperature, becomes more or less dormant early in the autumn. Owing to this characteristic, the growth of Turkistan and Ladak alfalfas at Riverside, Calif., is greatly retarded as early as September and is much delayed in the spring. These alfalfas, therefore, are not suitable for commercial production in the Southwest. In view of this fact it seems desirable either to find a selection from one of these alfalfas with the dormant character less pronounced or to search for bacterial wilt resistance in the less winter-hardy types of alfalfa. With this purpose in mind a study was undertaken to compare the relative resistance to bacterial wilt of a number of different lots of alfalfa under California conditions. The present paper is a progress report of the work on this project during the 6 years 1930-35.

## METHODS

Most of the seed lots studied were supplied by H. L. Westover, of the Bureau of Plant Industry, United States Department of Agriculture, Washington, D. C. Samples of locally grown seed also were included. The seed was sown in the greenhouse and in rows in the field. The plants usually were 4 to 8 months old when inoculated. The roots were dug carefully, so that a section of the taproot 4 or more inches long remained intact. Besides the broken roots, additional wounds for the entrance of the bacteria were provided by scraping some of the corky layer from the surface of one side of the taproot. This method of wounding the plants was similar to the one described by Jones (2). Usually a small segment of the lower end of the taproot was cut off just before inoculation. The plants were inoculated by immersing the roots for several minutes in a heavy bacterial suspension. Bacteria from fresh cultures of a recent isolation were used. All the bacteria that could be scraped and rinsed conveniently from about 25 agar slant cultures were used for

<sup>1</sup> Received for publication Dec. 12, 1935; issued April 1936. Cooperative investigations between the California Agricultural Experiment Station and the Division of Forage Crops and Diseases, Bureau of Plant Industry, U. S. Department of Agriculture.

<sup>2</sup> Reference is made by number (italic) to Literature Cited, p. 555.

each gallon of suspension. The plants usually were set in the ground as soon as possible after inoculation, although in one instance the inoculations were made several days prior to replanting with equally good results.

The plot at Delhi, Calif., where much of the early work was done, was on light sandy soil on which badly wilted plants had grown the previous season, while that at Davis, Calif., used later, was on a heavy black soil. The plants were inoculated either in late autumn or early spring and were allowed to grow undisturbed for several months to a year, more commonly the latter, before they were dug and examined for the presence of bacterial wilt. The taproots were cut off just above the broken end, usually 6 to 12 inches below the crown, and the presence or absence of the characteristic yellowing due to bacterial wilt was determined. Plants which showed no top symptoms of bacterial wilt and whose roots were free of the characteristic yellow color were classed as healthy and the remainder as diseased. In some instances samples of diseased roots were placed in a fixing solution and later studied to confirm the field diagnosis. After the plants were examined those classed as healthy were again wounded, inoculated, and planted and allowed to grow another 6 months or a year. If any plants remained healthy after the second treatment, they were transferred to the greenhouse for seed production. In one case all of the plants in the plot that appeared to be healthy were permitted to go to seed the latter part of the summer following the second inoculation, the tops of the plants showing disease being kept cut to prevent blossoming and crossing with the others. The seed thus obtained was used in an experiment the results of which are shown later. (See table 3.)

## EXPERIMENTAL DATA

### TESTING UNSELECTED SEED LOTS

The results obtained from inoculating plants of 38 different seed lots of alfalfa are recorded in table 1. As shown in column 3, varying numbers of plants of the different lots were used. These plants were grown in rows in a field at Riverside, Calif., from seed sown March 12, 1930. They were dug October 23 and 24, 1930, inoculated, wrapped carefully in newspaper, packed in boxes and shipped by express to Delhi, Calif., where they were planted on October 27 to 29, 1930. They grew well for a time, but after a few months began dying rapidly from bacterial wilt. The percentage of plants still healthy in October 1931 is given in table 1, column 4.

Table 1, column 5, shows the number of plants that were judged to be worthy of further trial in October 1931. These plants for the most part were healthy, although a few had slight bacterial wilt infections. All of them were reinoculated and replanted as previously described. The results of this second inoculation and the additional year of growth on badly infested soil are evident from the figures in column 6, table 1. Four of the lots were 100-percent dead or diseased by the end of the first year, and many others were only slightly less seriously affected. Twenty of the reinoculated plants were 100-percent affected by the end of the second year. Only an occasional plant had survived and was apparently healthy. It will be noted that greater percentages of

the Turkistan, Hardistan, and Ladak plants were left than of any of the others. A few plants of the less hardy alfalfas, such as Hairy Peruvian and Kansas Common, remained. It is quite evident that a single inoculation did not serve to eliminate all of the susceptible plants. This observation confirms those of Jones (3) and Peltier and Tysdal (8).

TABLE 1.—Relative resistance of different seed lots of alfalfa to bacterial wilt, as indicated by percentage of plants uninfectd after 1 and 2 inoculations and by percentage of original number of plants still healthy at end of second year (1930-32)

Variety or source	Accession no. <sup>1</sup>	Plants inoculated October 1930	Plants uninfectd October 1931	Plants reinoculated October 1931	Plants uninfectd October 1932	Plants uninfectd after 2 inoculations
		Number	Percent	Number	Percent	Percent
Arabian.....		161	0.00	0		
India.....	Comm 2470.....	216	00	0		
Do.....	Comm 1724.....	167	00	0		
Kansas Common.....	F. C. 15940.....	211	.00	0		
Hairy Peruvian.....	F. C. 15836.....	221	.45	9	11 11	0.45
Italian.....	Comm 2004.....	213	.47	6	.00	.00
South African.....	S. P. I. 48094.....	212	.47	7	.00	.00
Arabian.....		210	.48	2	.00	.00
Spanish.....	Comm 2005.....	194	.52	5	.00	.00
French.....	F. P. I. 81489.....	193	.52	10	.00	.00
Arizona Common.....	F. C. 15837.....	183	.55	2	.00	.00
Grimm.....	N. & R. 444.....	178	.56	15	.00	.00
South African.....	F. C. 14204.....	210	.95	9	.00	.00
Smooth Peruvian.....	Comm 2274.....	203	.99	2	.00	.00
Argentine.....	Comm 2346.....	199	1.01	8	.00	.00
Do.....	F. C. 15966.....	193	1.04	2	.00	.00
Hardigan.....	Comm 2621.....	192	1.04	8	12.50	.52
Italian.....	F. C. 15657.....	175	1.14	4	.00	.00
California Common (Hemet).....		188	1.60	9	.00	.00
Provence.....	S. P. I. 21195.....	201	1.99	10	.00	.00
French.....	F. P. I. 81488.....	193	2.07	10	.00	.00
Dakota Common.....	F. C. 16083.....	221	2.26	21	.00	.00
Kansas Common.....	N. & R. 435.....	229	2.62	10	10.00	.44
Argentine.....	Comm 64153.....	198	3.03	9	.00	.00
Bebensee.....		189	3.17	6	.00	.00
Lebeau.....	Comm. 2622.....	217	4.15	16	6.25	.46
Cossack.....	Comm. 2441.....	209	4.78	9	.00	.00
Provence.....	F. C. 15744.....	225	4.89	12	.00	.00
Hairy Peruvian.....	N. & R. 437.....	196	6.63	13	7.69	.51
Turkistan.....	Comm 2674.....	181	7.73	9	11.11	.55
Do.....	191 <sup>2</sup> .....	126	9.52	9	22.22	1.59
Do.....	S. P. I. 31811.....	129	12.40	16	18.75	2.33
Hardistan.....		208	18.27	22	13.64	1.44
Ladak.....		171	20.47	31	16.13	2.92
Turkistan.....	S. P. I. 20988.....	178	26.97	40	15.00	3.37
Do.....	F. C. 15754.....	174	30.46	48	4.17	1.15
Do.....	Comm. 2230.....	68	30.88	18	5.56	1.47
Do.....	F. C. 14786.....	125	43.20	48	12.50	4.80

<sup>1</sup> F. C. indicates accession numbers of the Division of Forage Crops and Diseases, S. P. I., Division of Seed and Plant Introduction (since changed to F. P. I., Division of Plant Exploration and Introduction), N. & R., New and Rare Field Seed Distribution, Comm., seed purchased from seedsmen.

<sup>2</sup> Accession number of the California Agricultural Experiment Station.

Seed of other lots of alfalfa was sown in rows in the field at Davis, Calif., in April 1931, and the plants were inoculated and planted at Delhi in October 1931. The data obtained from this experiment are recorded in table 2. Here again four of the lots, namely, Morocco F. P. I. 88912 and three Spanish lots, failed to survive the first inoculation. Many others fared little better. Again the Turkistan, Hardistan, and Ladak lots contained more resistant individuals than any of the others except Iran (Persia) F. P. I. 86362. Since most Persian strains show relatively low resistance, the higher degree of resistance

displayed by F. P. I. 86362 raises a question as to whether it came originally from Turkistan.

The relative resistance to bacterial wilt of common alfalfa from Hemet, Riverside County, Calif., and from Modoc County, Calif., so far as known, has not been reported previously. Out of 188 plants of alfalfa from Hemet inoculated in October 1930 (table 1) none survived the 2-year test, while of the 191 plants first inoculated in October 1931 (table 2) only 2.09 percent remained healthy at the end of the second year. A single plant of the alfalfa from Modoc County withstood the double inoculation.

Arizona Common and Hairy Peruvian alfalfas, which are commonly grown in California as well as in neighboring States, proved to be very susceptible in these tests as well as in those previously reported by other workers. Apparently the alfalfa commonly grown in California, regardless of whether the seed comes from Modoc or Riverside Counties or from Arizona, is almost certain to prove susceptible to bacterial wilt.

TABLE 2.—*Relative resistance of different seed lots of alfalfa to bacterial wilt, as indicated by percentage of plants uninfectd after 1 and 2 inoculations and by percentage of original number of plants still healthy at end of second year (1931-33)*

Variety or source	Accession no	Plants inoculated October 1931		Plants uninfectd October 1932		Plants re-inoculated October 1932		Plants uninfectd October 1933		Plants uninfectd after 2 inoculations
		Number	Percent	Number	Percent	Number	Percent	Number	Percent	
Morocco.....	F. P. I. 88912.....	68	0.00	0	0.00	0	0.00	0	0.00	0.00
Spanish.....	F. P. I. 89887.....	200	.00	0	.00	0	.00	0	.00	.00
Do.....	F. P. I. 89886.....	205	.00	0	.00	0	.00	0	.00	.00
Do.....	F. P. I. 89882.....	204	.00	0	.00	0	.00	0	.00	.00
California Common (Modoc County).....		210	.48	1	100.00					.48
French.....	F. C. 19274.....	140	.71	1	100.00					.71
Spanish.....	F. P. I. 89635.....	203	.99	2	50.00					.49
French.....	F. C. 19273.....	154	1.30	2	.00					.00
Kansas Common.....	F. C. 19180.....	202	1.98	4	25.00					.49
California Common (Hemet).....		191	4.71	9	44.44					2.09
Morocco.....	F. P. I. 88936.....	201	2.49	5	40.00					1.00
Spanish.....	F. P. I. 89884.....	170	2.94	5	40.00					1.18
Morocco.....	F. P. I. 88914.....	33	3.03	1	.00					.00
Spanish.....	F. P. I. 89846.....	199	6.03	12	33.33					2.01
Iran (Persia).....	F. P. I. 86361.....	197	8.63	17	58.82					5.08
Hardistan.....		192	18.75	35	54.29					9.90
Turkistan.....	F. P. I. 85751.....	206	20.87	43	46.51					9.70
Ladak.....	F. C. 15988.....	94	23.40	22	36.36					8.51
Turkistan.....	F. C. 19300.....	206	28.16	58	56.90					16.02
Do.....	F. C. 19316.....	209	34.93	71	73.24					24.88
(Iran) Persia.....	F. P. I. 86362.....	42	35.71	14	78.57					26.19

#### TESTING SINGLE-PLANT PROGENY

Data obtained by inoculating the progeny of single plants are given in table 3. Each entry in column 3, combined with the corresponding entries in columns 1 and 2, is the designation of a single plant. These plants include most of those surviving the second inoculation shown in column 7, table 1. As previously stated (p. 548), the seed was obtained during the summer of 1932 by open pollination. This seed was sown in the greenhouse at Davis in the early winter, and the plants were inoculated and transplanted to the field on April 25, 1933. When the plants were examined on November 2, 1933, there were

almost no top symptoms of bacterial wilt and the stand was considered almost perfect. The plants remaining healthy were reinoculated, replanted, and permitted to grow until November 1934. The final results are shown in columns 7 and 8, table 3.

These data are of considerable interest, since they show the reaction to bacterial wilt of plants whose parents withstood a very severe test for resistance. The percentage of healthy plants remaining after the second inoculation varied from 0 to 100, being in general considerably higher than in previous experiments. For example, in table 1 Turkistan F. C. 14786 shows 43.20 and 12.50 percent, respectively, of healthy plants surviving the first and second inoculations. In table 3 is shown the reaction of the progeny of six different plants of Turkistan F. C. 14786. Here the percentage of healthy plants after two inoculations varied from 100 in nos. 4 and 8 to 38.54 in no. 16, the average percentage of the progenies of all six of the plants remaining healthy being 64.57. On the other hand, Turkistan 191-2 after the first inoculation showed the same percentage of healthy plants (22.22) as the original stock when inoculated the second time (table 1, column 6), but no plants in the former survived the second inoculation. Turkistan 191-1 likewise failed to show any significant increase in resistance over its original stock.

TABLE 3.—Relative resistance of the progeny of individual plants which had survived 2 inoculations

[The seed was produced by open pollination]

Variety or source	Accession no	Plant selection no	Plants inoculated April 1933	Plants uninfectd November 1933	Plants reinoculated November 1933	Plants uninfectd November 1934	Plants uninfectd after 2 inoculations
			<i>Number</i>	<i>Percent</i>	<i>Number</i>	<i>Percent</i>	<i>Percent</i>
Turkistan.....	191.....	1	61	37.70	22	31.82	11.48
		2	9	22.22	2	.00	.00
		4	2	100.00	2	100.00	100.00
		6	25	96.00	22	63.64	56.00
		8	1	100.00	1	100.00	100.00
		10	7	57.14	4	75.00	42.86
	F. C. 14786.....	13	30	80.00	21	71.43	50.00
		16	96	77.08	67	55.22	38.54
		1	16	87.50	12	83.33	62.50
		5	23	52.17	12	75.00	39.13
		1	132	52.27	67	43.28	21.97
		10	3	33.33	1	100.00	33.33
	S. P. I. 20988.....	14	38	78.95	26	30.77	21.05
		18	1	.00			
		1	8	37.50	2	50.00	12.50
	S. P. I. 31811.....	2	32	81.25	25	24.00	18.75
		4	110	66.36	68	55.88	34.55
		1	37	67.57	24	62.50	40.54
		2	17	47.06	6	83.33	29.41
Ladak.....		3	69	53.62	35	48.57	24.64
		8	26	80.77	20	65.00	50.00
		11	40	47.50	19	52.63	25.00
		1	19	47.37	9	11.11	5.26
Kansas Common.....	N. & R. 435.....	1	190	15.79	29	20.69	3.16
Lebeau.....	Comm. 2622.....	2	1	50.00	1	100.00	50.00
Hardigan.....	Comm. 2621.....	1	3	66.67	1	100.00	33.33
Hary Peruvian.....	F. C. 15836.....	1					

Similar comparisons of other plants and their respective progenies, as listed in the tables, show that resistance to bacterial wilt is transmitted to the offspring in different degrees by different resistant plants. In some instances there seems to be no increase in resistance

in the first generation over the seed stock, while the reverse is true in other cases. Obviously the fact that a plant has survived two inoculations and 2 years' growth on infested soil does not necessarily mean that it will produce progeny of a higher degree of resistance. A similar conclusion has been reached by Jones (2), Brink, Jones, and Albrecht (1), and Peltier and Tysdal (8).

In this connection tables 4 to 6 are of interest. Table 4 is designed to show more clearly the comparative resistance of the seed stock and the first generation of certain plants of some seed lots tested. The percentages of plants of each generation remaining healthy after two inoculations are given in columns 4 and 5. In table 4, columns 1 and 2, the first item in each group of plants recorded represents the stock from which the other plants were obtained. For example, the original stock of Turkistan 191, whose resistance is shown in table 1, is compared with the progeny of 191-1 and 191-2, two plants selected from this stock. The original number of plants of 191 set out, as shown in table 1, column 3, was 126. Of these only 2 (191-1 and 191-2) survived the second inoculation, or, in other words, 1.59 percent remained healthy (column 4, table 4). The percentages of the progenies of these two plants that were still healthy after two inoculations are given in column 5, table 4. In like manner Turkistan alfalfas F. C. 14786, F. C. 15754, S. P. I. 20988, S. P. I. 31811, and several other seed stocks are compared with their respective progenies. The increase in resistance is quite striking in some cases and nil in others.

TABLE 4.—Comparison of the resistance of certain seed lots with that of their progeny

[Progeny from open-pollinated seed]

Variety or source	Accession no.	Plant selection no.	Percentage of healthy plants after 2 inoculations		Variety or source	Accession no.	Plant selection no.	Percentage of healthy plants after 2 inoculations	
			Original stock	Progeny				Original stock	Progeny
Turkistan	191	1	1.59	11.48	Turkistan	S P I 31811	1	2.33	12.50
		2		.00			2		18.75
		3					3		34.55
		4					4		34.55
	F C 14786	4		100.00	Ladak		1	2.92	40.54
		6		56.00			2		29.41
		8		100.00			3		24.64
		10		42.86			8		50.00
	F C 15754	13		50.00	Kansas Common	N & R 435	11	44	5.26
		16		38.54			1		3.16
		1	1.15	62.50			2		52
		5		39.13			1	45	33.33
	S P I 20988	3	3.37	21.97	Hardigan	Conum 2621	1		50.00
		10		33.33			1		50.00
		14		21.05			1		50.00
		18		00			1		50.00
		1		21.97	Hairy Peruvian	F C 15336	1		50.00
		10		33.33			1		50.00
		14		21.05			1		50.00
		18		00			1		50.00

Table 5 gives the reaction to bacterial wilt of progenies of some of the plants shown in table 4, but from seed grown in the greenhouse and hence selfed. The percentages of healthy plants surviving the two inoculations are given in column 8. These figures enable one to compare the relative resistance of the progenies obtained by close pollination (table 5) with those secured by open pollination (tables 3 and 4).



as well as with the original seed stock (table 1). In general, there was considerable increase in resistance in the progenies of these selected plants when close-pollinated over that of the original stock from which the parent plants were selected. The progenies of the parent plants when open-pollinated also showed more or less increase in resistance. However, between the two sets of progenies there was no consistent difference that might be attributed to the difference in the method of pollination.

TABLE 5.—Resistance to bacterial wilt of progeny of resistant plant selections

[Progeny from close-pollinated seed]

Variety or source	Accession no.	Plant selection no	Plants inoculated April 1934	Plants uninfectd November 1934	Plants re-inoculated November 1934	Plants uninfectd May 1935	Plants uninfectd after 2 inoculations
			<i>Number</i>	<i>Percent</i>	<i>Number</i>	<i>Percent</i>	<i>Percent</i>
Turkistan	F C 14780	127	56	50.00	26	92.31	42.86
		128	122	38.52	47	82.98	31.97
		143	13	69.23	5	87.50	53.85
		131	39	48.72	19	78.95	38.40
		132	64	45.31	27	100.00	42.19
	S P I 20985	137	29	24.14	8	37.50	10.34
		139	33	51.52	15	53.33	24.24
		144	20	20.00	4	100.00	20.00
		138	29	17.24			
		141	52	65.38	34	88.24	57.69
	S. P I 31811	126	177	35.03	62	93.55	32.77
		135	71	38.03	28	67.86	26.76
Hardistan	F C 15754		45	51.11	21	66.67	31.11
Ladak		52	55	63.64	33	75.76	45.45
		53	8	50.00	4	75.00	37.50
Hardigan	Common 2621	49	73	54.79	39	20.51	10.96
California (Hemet)			28	10.71	3	66.67	7.14
Hairy Peruvian (control)			9	22.22	2	.00	.00
Grizum (control)	F. C. 22047		16	.00	0	.00	.00

F. R. Jones contributed some alfalfa plants which he had selected for their resistance to bacterial wilt at Madison, Wis. These were planted in the greenhouse at Davis, Calif., and seed was obtained from most of them. The plants from this seed were inoculated in the usual way. The inoculations were made in April and November 1934, and the plants were examined in November 1934 and May 1935, respectively. The percentage of the progenies from each plant remaining healthy is given in table 6. The source of the parent plants is shown in table 6, column 1. These plants were presumably of the Common variety, 1301-1, 1304-8, and F. C. 15749 being recorded by Jones as of the semihardy type. Some of these plants produced progeny of a low degree of resistance, as shown in column 8, while the reverse is true of others. A better understanding of the potential value of a line in the nonwinter-hardy group, which shows 50 percent or more of resistant plants, is obtained by a comparison with those of the same group; for example, with the California Common grown in the vicinity of Hemet and Hairy Peruvian, listed in table 5, and with other nonhardy alfalfas, listed in tables 1 and 2. Very commonly the plants belonging to the nonhardy group are 100-percent affected as a result of the first inoculation. Several of the plants sent by Jones, some of which are nonhardy, have produced progeny apparently equal in

resistance to the Turkistans and Ladak. The prospect of obtaining some valuable selections from these plants seems promising.

TABLE 6.—Resistance of the progeny of plants selected by F. R Jones at Madison, Wis.

[Selfed seed grown at Davis, Calif]

Variety and source of seed lot from which selections were made	Accession no.	Plant selection no	Plants inoculated April 1934	Plants uninfectd November 1934	Plants re inoculated November 1934	Plants uninfectd May 1935	Plants uninfectd after 2 inoculations	
			Number	Percent	Number	Percent	Percent	
Kansas Common	1225	48	124	21.77	25	28.00	5.65	
		101	55	12.73	7	42.86	5.45	
		102	46	30.43	13	100.00	28.26	
		111	74	56.76	41	73.17	40.54	
		3-108	22	50.00	11	81.82	40.91	
Montana Common	1300	3-109	89	21.35	18	66.67	13.48	
		3-110	60	75.00	39	79.49	51.67	
		1-39	30	56.67	17	76.47	43.33	
		2-121	124	16.94	20	50.00	8.06	
		2-105	45	48.89	20	80.00	35.56	
Kansas Common	1302	2-107	37	62.16	20	100.00	54.05	
		2-116	100	76.00	69	85.51	59.00	
		2-117	21	71.43	14	100.00	66.67	
		2-120	25	72.00	15	100.00	60.00	
		2-122	26	53.85	14	100.00	53.85	
	1303	2-124	59	40.68	21	80.95	28.81	
		3-118	77	76.62	58	74.14	55.84	
		2-47	19	78.95	15	73.33	57.89	
		2-103	21	28.57	7	57.14	19.05	
		2-106	18	11.11	2	50.00	5.56	
	1304	8-13	37	5.41	2	100.00	5.41	
		8-20	103	29.13	28	42.86	11.65	
		30	36	27.78	15	93.33	38.89	
		F. P. I. 88141	75	52.00	38	97.37	49.33	
		(1)-50	16	50.00	8	100.00	50.00	
Spain		F. P. I. 88143	56	22	9.09	2	50.00	4.55
		F. P. I. 89843	55	23	.00			.00
		F. P. I. 89631	54	3	.00			.00
		F. P. I. 89883	2-43R	115	21.74	24	62.50	13.04
		F. P. I. 89854	25	96	35.42	33	48.48	16.67
Portugal	F. P. I. 89904	3	82	53.66	42	54.76	28.05	
		5	91	42.86	38	47.37	19.78	
		11	26	19.23	4	100.00	15.38	
		14	93	55.91	52	50.00	27.96	
		(1)-22R	76	17.11	13	76.92	13.16	
Morocco	F. P. I. 89973	51	17	35.29	4	.00	.00	
Kansas Common	F. C. 15749	34	100	19.00	19	68.42	13.00	
Hairy Peruvian (control)			68	64.71	43	79.07	50.00	
Grimm (control)	F. C. 22047		9	22.22	2	.00	.00	
			16	.00	0	.00	.00	

<sup>1</sup> Accession number of Wisconsin Agricultural Experiment Station

## SUMMARY

Alfalfa plants produced from 59 different lots of seed from various sources have been tested for resistance to bacterial wilt at Delhi and Davis, Calif.

The plants were inoculated twice and grown in field plots for 6 months to 1 year following each inoculation. Seed was obtained from the plants which remained healthy and the resistance of the new generation was tested.

The results confirm those of other workers in that they show that Turkistan, Hardistan, and Ladak alfalfa seed lots contain the highest percentages of resistant individuals. A few plants of some of the other seed lots survived the two inoculations. The progenies of these are being tested.

Iran (Persia) F. P. I. 86361 and F. P. I. 86362 contained more resistant individuals than most of the others, with the exception of Turkistan, Hardistan, and Ladak, and, since most lots from Iran have shown relatively low resistance, the question arises as to whether these lots came originally from Turkistan.

For the most part, progenies of plants that survived two inoculations gave a somewhat higher percentage of resistant individuals than did the original stock. Some of the progenies were no more resistant than the stock from which the original selections were made, while the reverse was true in other cases, showing that resistance is transmitted to the offspring in varying degrees by different plants.

Plants from 38 different individuals selected by F. R. Jones, many of which were from the nonhardy group of alfalfas, were tested. The progenies of some of these plants contained many individuals which survived two inoculations.

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## INFLUENCE OF TEMPERATURE AND OTHER FACTORS ON THE MORPHOLOGY OF THE WHEAT SEEDLING<sup>1</sup>

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### INTRODUCTION

The morphology of the wheat seedling (*Triticum vulgare* Vill.<sup>2</sup>) is affected by both soil temperature and light during the early stages of growth. Two of the more prominent and important seedling characters determined during early growth are the length of the coleoptile and the position or depth of the crown in the soil. The coleoptile, though its life is short, is of prime importance to normal germination. It is also the organ through which organisms causing some of the more serious plant diseases enter the seedling. The location of the crown in relation to the soil surface can readily influence tillering, crown-root development, heaving, and winter injury. Another seedling character affected by environment is the seminal root system.

The present study, conducted in the greenhouses at the Arlington Experiment Farm, Rosslyn, Va., near Washington, D. C., shows the influence of four constant soil temperatures on the seedling development of a spring and a winter wheat sown at two depths. In addition, some observations on certain factors influencing the seminal root frequency have been made.

### REVIEW OF LITERATURE

The influence of depth of seeding on the coleoptile length of wheat was observed by Percival (9).<sup>3</sup> He also has described the two root systems and the factors influencing their formation and extent.

Dickson (2) noted the effect of soil temperatures on both coleoptile length and elongation of the subcrown internode of two varieties of wheat.

Kossowitsch (5) found that plants grown in shaded areas had higher crowns than those grown in full sunlight. He also showed in plant-house experiments that high temperatures gave a similar result. In addition, he gives field data showing that winter-killed plants had much shallower crowns than the surviving plants.

Tavčar (12) states that the variety least damaged by cold has the vegetative point of its principal stalk the most deeply buried.

Gladkii and Lykhvar (4) believe that soil moisture (about 18 per cent) appears to be the main factor in the development of the under-

<sup>1</sup> Received for publication Oct. 5, 1935, issued May 1936.

<sup>2</sup> According to the rules of botanical nomenclature the name of this species is *Triticum aestivum* L., but as *T. vulgare* is in general use among agronomists and cereal pathologists and geneticists, the writers give preference to that name.

<sup>3</sup> Reference is made by number (italic) to Literature Cited, p. 568.

node or coleoptile tiller. From 10 to 50 percent of the heads can come from this node.

Friedberg (3) found varietal differences in the rate of coleoptile tillering. He believes the coleoptile tiller can be a factor in recovery from winter injury.

#### MATERIAL AND METHODS

Well-developed kernels of Hard Federation (C. I.<sup>4</sup> 4733) and Turkey (C. I. 1558), both from Moccasin, Mont., were used. These varieties differ greatly in growth habit. Hard Federation is an early spring wheat with white kernels, introduced from Australia by the United States Department of Agriculture in 1915, and Turkey is a midseason, hardy, hard red winter wheat probably introduced into Kansas in 1873 by Russian Mennonites (1).

The seeds were sown in galvanized-iron cans 8 inches in diameter and 9½ inches deep, filled 7½ inches with a uniform loam. The desired moisture content was maintained by triweekly weighings and additions of water. Soil-surface evaporation was reduced by a coarse granular cork mulch.

For each variety there were 16 cans at temperatures of 12°, 16°, and 24° C. and 20 cans at 20° C. These were maintained constantly by thermostatic control. The mechanism of the tanks (controlling the temperatures) is described in detail by Leukel (6). Twelve seeds were sown to a can, and later the plants were thinned to four seedlings.

Two seedlings, one made on December 5, 1928, and another on February 6, 1929, were studied. The most important difference between the two seedlings, other than date with consequent light effects, was the depth of seeding. In the earlier sowing the seeds were 18 mm and in the later they were 45 mm below the top of the cork mulch.

The principal measurements made were length of coleoptile, length of subcrown internode, number of coleoptile tillers, number of tillers, and number of crown roots. The tillers and crown roots were counted when the plants were 8 weeks old in the first seeding and approximately 6 weeks old in the second.

#### EXPERIMENTAL DATA

##### GERMINATION

Germination of both Hard Federation and Turkey was most rapid at 24° C. in both seedings. At each lower temperature the germination period increased. As shown in table 1, no significant differences in the rate of germination between Hard Federation and Turkey occurred at any temperature.

##### EFFECT OF TEMPERATURE AND DEPTH OF SEEDING ON LENGTH OF COLEOPTILE

Soil temperature exerted a decided influence on the length of the coleoptile of the plants sown 18 mm deep (table 1). The average length of the coleoptile of both varieties grown at 20° C. was approximately 10 percent less than at 24° and from 25 to 28 percent less than at 16°. There were no significant differences in coleoptile length

<sup>4</sup> C. I. refers to accession number of the Division of Cereal Crops and Diseases, formerly Office of Cereal Investigations.

between the plants grown at 16° and at 12°. When the seed was sown 45 mm deep, temperature differences had little effect on coleoptile length, as the greatest difference due to temperature in either variety was less than 6 percent.

TABLE 1.—Effect of temperature and depth of seeding on plant characters of Hard Federation and Turkey wheats grown in controlled-soil-temperature tanks at the Arlington Experiment Farm, Rosslyn, Va.

SEED SOWN 18 MM DEEP							
Variety and soil temperature (°C)	Planting to emergence	Coleoptile length	Subcrown-internode length	Planting to tillering	Tillers per plant	Plants with coleoptile tillers	Crown roots per plant
Hard Federation	Hours	Mm <sup>1</sup>	Mm <sup>1</sup>	Days	Number <sup>1</sup>	Percent	Number <sup>1</sup>
24.....	92	54 9±0 57	31 7±0 51	33	2 9±0 06	0	4 2±0 20
20.....	104	49 3± 57	26 2± 38	28	3 3± 07	0	6 3± .21
16.....	178	41 4± 45	20 3± 33	28	3 8± 06	2	6 9± .15
12.....	211	42 3± .46	12 3± 32	33	3 1± 06	4	6 3± .15
Turkey							
24.....	92	45 9± 47	22 3± 42	23	8 5± 28	0	6 9± .20
20.....	104	40 9± .40	19 8± .30	21	10 1± 26	6	7 5± .29
16.....	166	33 2± .31	13 5± .30	23	12 7± 19	56	5 4± .14
12.....	211	32 5± 24	3 9± 21	24	10 3± .16	79	3 4± .10
SEED SOWN 45 MM DEEP							
Hard Federation							
24.....	96	61 5±0.33	33. 5±0 51	26	2 7±0.07	2	5. 6±0.11
20.....	122	61. 8± .27	33 0± .43	26	2 7± .05	0	5 8± .12
16.....	161	61 5± 28	27 4± 42	25	2 6± 05	0	3 7± .12
12.....	218	58 6± .25	26. 2± .35	34	2 7± 05	3	2 8± .09
Turkey							
24.....	96	53 0± 22	24 7± 62	19	4. 8± .16	0	3 8± .18
20.....	120	54 3± 22	21. 6± 45	19	5. 0± .12	4	2 4± .08
16.....	154	53 3± 17	15 7± 42	20	6 5± .17	48	1. 8± .09
12.....	216	51 4± .16	14 6± 41	26	5 6± .09	87	1. 2± .08

<sup>1</sup> The number following the plus-or-minus sign represents the probable error.

The effect of depth of seeding, or lack of light, on the coleoptile length is shown by comparing the data from the first and second seedlings (table 1). At 24° C. the coleoptile length of the shallow-sown Hard Federation was 11 percent less than that of the deeper sown; at 20° it was 20 percent less, and at 16° it was 33 percent less. Turkey behaved similarly.

Hard Federation had a longer coleoptile than Turkey at all four temperatures, at both seeding depths. The varietal contrast was greater in the shallow seeding and increased inversely with temperature, ranging from 16 percent to 23 percent shorter for Turkey. When the seed was sown 45 mm deep, the varietal contrast differed but slightly with temperature, Turkey being only 12 to 14 percent shorter.

#### EFFECT OF TEMPERATURE AND DEPTH OF SEEDING ON LENGTH OF SUBCROWN INTERNODE

The location of the crown is dependent on the elongation of the internode or internodes between the basal attachment of the coleoptile and the first node of the crown. Normally, the latter is the node of the first foliage leaf, although in some cases it may be that of the second, third, or even fourth (fig. 1). The region between the coleoptile base and the crown comprises the subcrown internode or

internodes. The critical period determining the crown location, or the elongation of the subcrown internode, occurs soon after germination. Twelve days after sowing, or approximately 6 days after emergence, both at 20° and 24° C., the lower node of the crown of Hard Federation

could be identified with the naked eye and was approximately in its final position.

At the temperatures and seeding depths used in these experiments the plants crowned normally, i.e., the first crown node was that of the first foliage leaf. The subcrown region above the coleoptile attachment consisted, therefore, of a single internode, the elongation of which determined the position of the crown.

In both Hard Federation and Turkey, the length of the subcrown internode increased progressively from 12° to 24° C., at both seeding depths. Temperature effects on subcrown internode length, as on coleoptile length, were greatest in the shallow-sown plants. When seeds were sown 18 mm deep the subcrown internode of Hard Federation averaged 17 percent shorter at 20° than at 24°, and that of Turkey 11 percent shorter. The subcrown internode elongation of Hard Federation at 16° and at 12° averaged 36 and 61 percent shorter, respectively, than at 24°, whereas in Turkey the elongation at 16° and at 12° was 38 and 83 percent shorter, respectively.

Deep seeding increased the length of the subcrown internode of both varieties at all four temperatures (table 1), the differences being slight at 24° C. but increasing at the lower temperatures. At 12° the internode of Hard Federation was 53 percent shorter from the shallow



FIGURE 1—Wheat seedling showing development of the main crown at the second node rather than at the first.



seedling than from the deep seeding and that of Turkey 73 percent shorter.

Hard Federation crowned higher than Turkey at the four temperatures, in both seeding depths. The varietal contrast was sharp in all comparisons but most marked at 12°, especially in the 18 mm seeding, at which the crown of Turkey was 68 percent lower than that of Hard Federation. At the same temperature, Turkey, when sown 45 mm deep, crowned 44 percent lower than Hard Federation. At the two highest temperatures the difference was least, but still there was a minimum difference of 26 percent.

Hard Federation, when sown 18 mm deep and grown at 24°, 20°, and 16° C., crowned above the soil line, and Turkey behaved similarly at 24° and 20°. When the seed was sown 45 mm deep, the crown

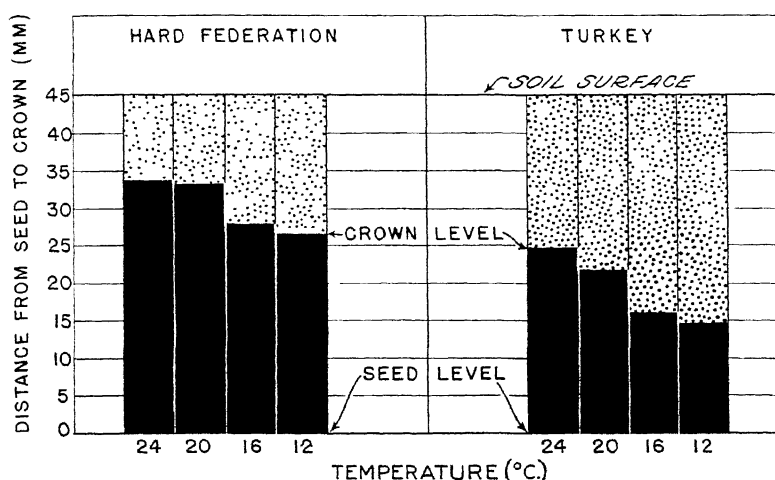


FIGURE 2.—Location of the crown with reference to the surface of the soil when the seed was sown 45 mm deep and grown at four constant temperatures

was well below the soil surface at all temperatures. The average position of the crown with reference to the soil surface at the deeper seeding is shown in figure 2.

#### EFFECT OF ENVIRONMENT AND VARIETY ON TILLERING

In the axil of each leaf, including the coleoptile and prophyll, there is a tiller bud. Inasmuch as each tiller has its prophyll, the extent of tillering is theoretically limited only by environment. However, it is generally accepted that varieties differ inherently in tillering proclivity.

In the writers' studies Turkey always tillered before Hard Federation. At the shallower seeding there was an average lapse of 23 days before the appearance of first tillers in Turkey and almost 31 days in Hard Federation. The corresponding intervals for the deeper seedings were 21 and 28 days. Hard Federation sown 18 mm deep and grown at 16° C. averaged 3.8 tillers per plant and Turkey averaged 12.7 tillers per plant. At all temperatures in both seedings (table 1), Turkey tillered more extensively than Hard Federation. In both

varieties, where differences were found, tillering was least at 24° and greatest at 16°.

The growth of the coleoptile axillary bud appears particularly sensitive to conditions during the early seedling development. When conditions are favorable the coleoptile axillary bud develops into the first tiller of the plant. Shortly afterward the tiller bud in the axil of the first seedling leaf develops into the second tiller, and the third tiller normally comes from the bud in the axil of the second leaf. The fourth tiller to appear may come either from the bud in the axil of the third leaf or from the prophyll axillary bud of the tiller in the



FIGURE 3—Wheat plant with (a) crown developed from coleoptile tiller and (b) main crown.

axil of the first foliage leaf. In some varieties the coleoptile tiller fails to develop under most conditions. Theoretically, it would appear advantageous to have varieties tending to develop a tiller from the coleoptile node. This would produce a plant with two crowns, as shown in figure 3. Injury by low temperature, heaving, winds, or grazing by stock could kill or severely check the main crown and still leave the other capable of producing a crop. The coleoptile tiller forms what is in effect a second plant with its crown usually located deeper in the soil than the main crown. The significance of this second crown has been little investigated.

If the coleoptile tiller is to compete with the main crown tillers and develop significantly it must have an early start. Such a condition existed when the seeds of Turkey were germinated at 12° C., as the

first tiller recorded in all pots was from the coleoptile axillary bud. The development of the coleoptile-node tiller is apparently determined by the availability of reserve food materials associated with the slow growth rate of the low-temperature environment.

As shown in table 1, Hard Federation rarely developed the coleoptile tiller at any of the four temperatures, the highest percentage being 4 at 12° C., with shallow seeding.

In Turkey, at 24° C., no coleoptile tillers were recorded; at 20° from 4 to 6 percent of the plants tillered from the coleoptile axil; while at 16° and 12° the percentage increased to approximately 50 and 80, respectively.

#### EFFECT OF ENVIRONMENT AND VARIETY ON EARLY FORMATION OF CROWN ROOTS

At the time counts were made more than 28 percent of the Hard Federation plants sown 18 mm deep at 24° C. had failed to develop functional crown roots. This was due to too high crowning. The formation of adventitious roots is very evidently closely associated with moisture. When plants grew so that the crown nodes came in contact with the moist soil, roots formed readily. Also, it was observed that crown roots formed even in the air at a sufficiently high degree of humidity. About 14 percent of the plants without crown roots had only the main stem, but the remaining 86 percent had one or more tillers. It is evident that formation of the first tillers may occur independently of crown-root functioning. The later degree of development of these tillers and of subsequent tillers, however, is undoubtedly closely associated with that of the adventitious crown roots.

The early development of tillers in excess of crown-root formation was most apparent in Turkey at 12° C. At the 18-mm depth of seeding the plants averaged 10.3 tillers but only 3.4 crown roots per plant. At the deeper seeding they averaged 5.6 tillers and only 1.2 crown roots. Hard Federation developed crown roots in excess of tillers under all conditions, but more at 12° in the 18-mm depth of seeding, averaging 6.3 crown roots and only 3.1 tillers (table 1).

#### EFFECT OF TEMPERATURE, DEPTH OF SEEDING, SIZE, AND PREMATURE HARVEST OF SEED ON NUMBER OF SEMINAL ROOTS

The seminal root system of wheat is not generally well understood, either in respect to number of roots or function. The information, however, is available. Wiggans (15) found that the seminal roots of common wheat varied from 1 to 6, and Locke and Clark (7) showed that the seminal root system may remain functional and carry the wheat plant through to maturity. McCall (8) found that there was a potentiality for as many as nine roots in the subcrown region which could be taken for seminal roots. However, the true seminal or seed roots are limited to a maximum of six: the primary, first and second pair of laterals, and a face root. At the coleoptile node, two, and occasionally three, adventitious roots may develop. These, however, should not be interpreted as seminal roots since their primordia are not present in the embryo. The development of the seminal roots is related to nutrition, and the full complement of six roots is rarely found.

Certain factors of possible influence in determining the number of seminal roots were investigated. These included temperature, depth of seeding, size of seed, and degree of seed maturity as determined by the interval between pollination and harvest.

Two seed sizes were used, the separation being made with a screen having 8 meshes per inch. The seed was germinated and grown at two temperatures—approximately 15° and 25° C.

The seedlings of Hard Federation differed but slightly in number of seminal roots when grown at 15° and at 25° C. The mean number of seminal roots at 15° was 3.5, and at 25° it was 3.4. The extreme numbers

were 3 and 6. The number of seminal roots of Turkey was decidedly increased, however, by the higher soil temperature, as shown in figure 4. The mean number of seminal roots at 15° was 3.1, and at 25° it was 4.0. More than 90 percent of the seedlings at 15° had only 3 seminal roots, as compared with but 28 percent at 25°.

When the plants were grown at a soil temperature of 12° to 15° C., seeding 75 mm deep resulted in fewer seminal roots of both Hard Federation and Turkey than did seeding 20 mm deep. Turkey, however, was less affected by depth of seeding than was Hard Federation. More than three times as many plants with four seminal roots were found in the 20-mm seeding depth of Hard Federation as in the 75-mm depth (fig. 5).

The number of seminal roots was greater in both Hard Federation and Turkey plants grown from large seed. Turkey showed less effect, however, than Hard Federation, the mean number of seminal roots for the former from large seed being 3.7 as compared with 3.5 for the small seed. Plants from large seed of Hard Federation averaged 3.6 seminal roots and those from small seed 3.2 seminal roots. Figure 6 shows the effect of seed size on the seminal-root frequency of Hard Federation. More than 80 percent

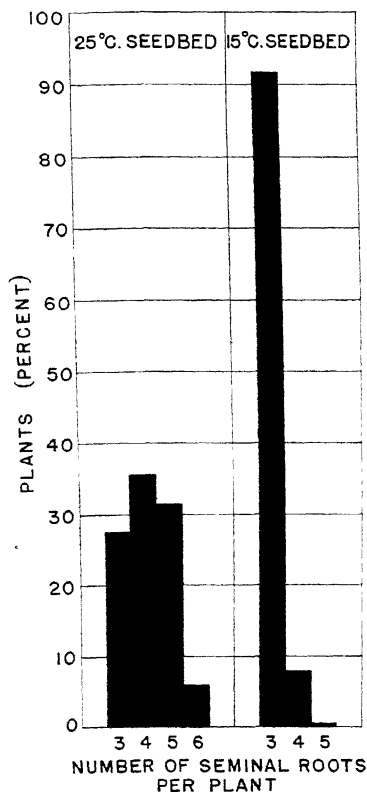


FIGURE 4.—Percentage of plants of Turkey wheat with different numbers of seminal roots when grown at soil temperatures of 25° and 15° C.

of the plants from small seed had but three seminal roots, while less than 50 percent of the plants from large seed had as few as three.

Robbins (10) found that the first pair of seminal rootlets appeared in the embryo about 4 weeks after pollination, and McCall (8) observed that wheat embryos varied in number of seminal-root primordia and that many did not show all six roots.

The number of seminal roots was determined on Purplestraw wheat seedlings from seed harvested 13, 20, and 30 days after flowering and cured in the shock. The results are shown in figure 7. The seed harvested 13 days after flowering produced about 50 percent of plants with three seminal roots and 15 percent with four seminal roots. The remainder were one- and two-root plants. The seed

harvested 20 days after flowering produced plants more than 60 percent of which had four seminal roots, and the seed harvested 30 days after flowering produced almost 70 percent of plants with five seminal roots. That these three seed lots differed decidedly in seminal-root production is evident, but whether these differences are due to the ages of embryos or to differences in food reserves is unknown.

#### COLEOPTILE-NODE ROOTS

The coleoptile-node roots, arising from just above the coleoptile node, are easily mistaken for seminal roots because in size and position they are so close to the true seminal roots. The number of the coleoptile-node roots appears to be related to food reserves. The relation of seed size, seeding depth, and age of embryo from pollination to the number of the coleoptile-node roots is shown in figure 8.

The number of coleoptile-node roots was much less from small seed and deep seeding, the effects of these conditions being about equal. Seed harvested 13 days after flowering produced plants with but few coleoptile roots as compared with seed harvested 20 and 30 days after flowering.

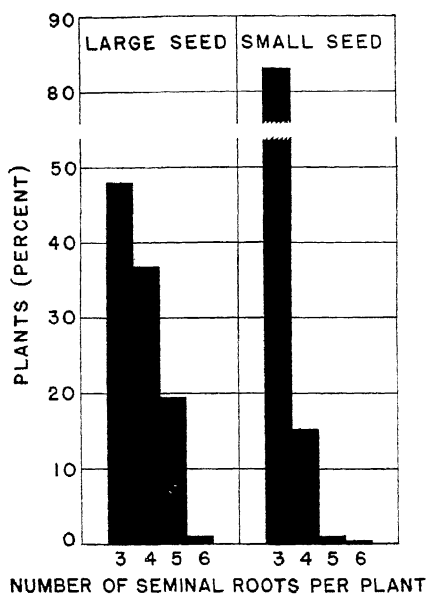


FIGURE 6—Percentage of plants with different numbers of seminal roots from large and small seed of Hard Federation wheat.

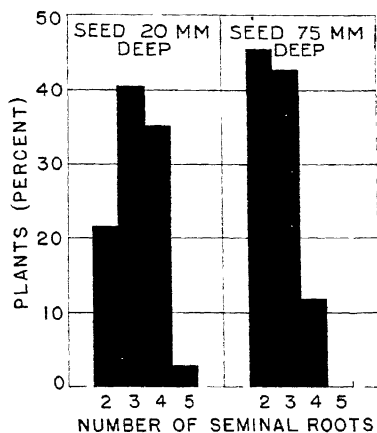


FIGURE 5—Percentage of plants with different numbers of seminal roots from seed of Hard Federation wheat sown 20 mm and 75 mm deep.

#### DISCUSSION

Temperature and depth of seeding both have a marked influence on the morphology of the seedling wheat plant. When the seed was sown only 18 mm deep, much longer coleoptiles were produced at 20° and 24° than at 12° and 16° C. Deeper seeding, at 45 mm, however, was an even more potent factor than high temperature in increasing coleoptile length, both Hard Federation and Turkey having longer coleoptiles at 12° in the deeper seeding than at 24° in the shallow seeding. The effect of deep seeding on the length of the subcrown internode showed a similar trend, but temperature here apparently exerted more influence. A morphological study of either of these two characters without consideration of the seeding depth would be incomplete.

The effect of the position of the crown with relation to the soil surface on the later behavior of the plant has been little investigated. Locke and Clark (7) have shown that in dry regions, when the crown lies in dry soil, crown roots do not form. This was also noted by Webb and Stephens (14). A high-crowning variety, or environmental conditions that result in high crowning, would emphasize this reaction. Kossowitsch (5) cites field measurements showing that the plants that were winter-killed had much shallower crowns than those that survived. The wide differences between Hard

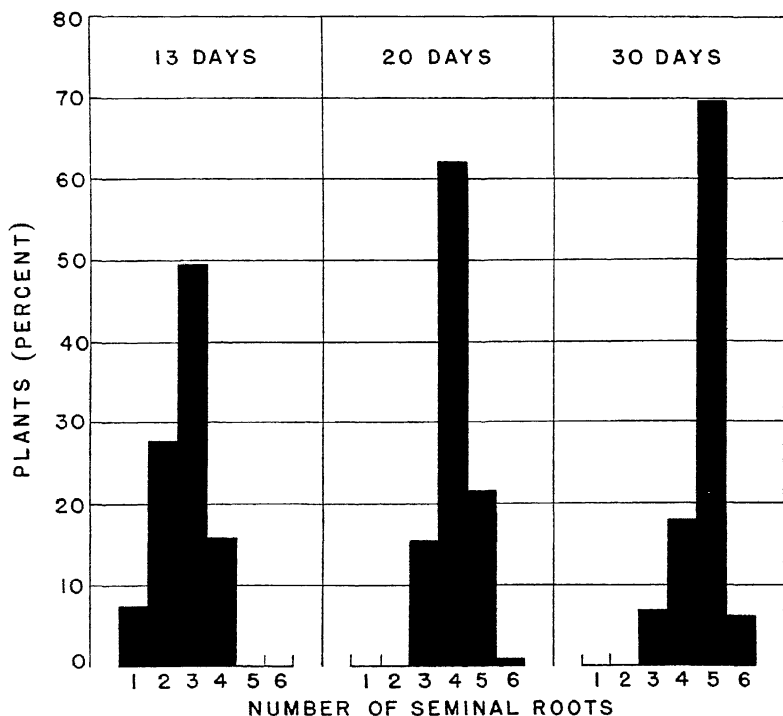


FIGURE 7—Percentage of Purplestraw seedlings with one to six seminal roots from seed harvested at three intervals after flowering.

Federation and Turkey in crowning habit, shown in these experiments and also by Webb and Stephens (14), indicate clearly that variety plays an important role.

The results here presented suggest that certain varietal differences in seedling wheat plants are largely determined by the degree of response to the temperature factor. The detrimental effects of too early sowing of winter wheat at a time when soil temperatures are still relatively high may well be caused, in part at least, by the effect of high temperature on crown position. The shallower crowns may be placed in drier soil, resulting in poor crown-root development. Later, cold injury may result from less protection or from poor root anchorage. These same factors would also be operative in periods of drought.

At favorable temperatures, Turkey possesses a decided tendency to develop a tiller and second crown from the coleoptile axillary bud. The crown resulting from this is usually located deeper in the soil than the main crown and, according to the observations of Kossowitsch (5), is less liable to winter injury. This second crown is therefore an additional safeguard. An analysis of varieties, both from the standpoint of temperature reactions in subcrown-internode elongation and in tendency to development of the coleoptile tiller, would seem useful as a basis for further improvement.

The slow development of the crown root system of Turkey may be one major reason why this variety often shows greater winter injury or killing in the eastern humid region than some of the varieties known to be less hardy at low temperatures. Heaving of plants from the ground is common, and in many years may be more serious than injury from low temperatures alone.

The importance of the seminal roots during the entire life cycle of the wheat plant has been well demonstrated by Simmonds and Sallans (11) and by Todaro (13). Whether it is important that the young seedling should have more than three seminal roots is entirely speculative in the light of present knowledge. On the basis of vigor, it could be assumed that a greater number is preferable. If so, this can be assured through seed quality, as measured by size and maturity.

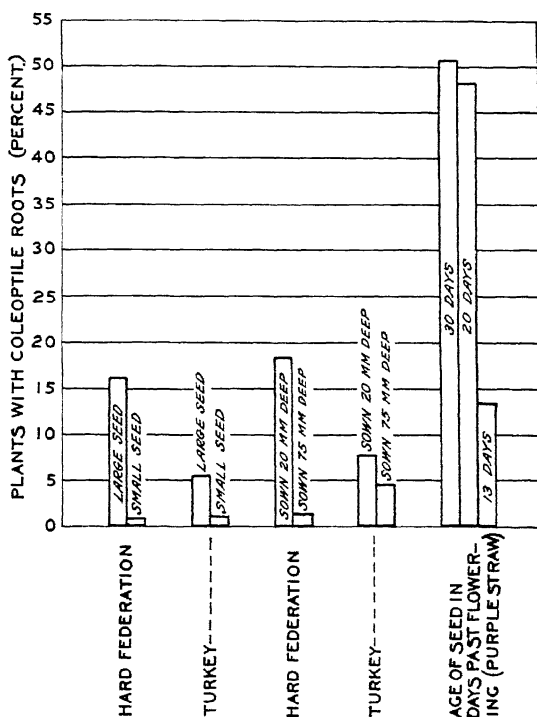


FIGURE 8.—Effect on number of coleoptile-node roots of the size of seed, depth of seeding, and age of seed from flowering.

#### SUMMARY

Temperatures of 24° and 20° C. increased the length of the coleoptiles of seedlings of Hard Federation and Turkey wheat as compared with temperatures of 16° and 12°. Deep seeding (45 mm) was more effective, however, than temperature in increasing the coleoptile length.

The length of the subcrown internode was also increased by both the high temperatures and the deeper seeding. In all comparisons, Hard Federation had a longer coleoptile and a longer subcrown internode than Turkey.

Coleoptile tillering seldom occurred at 24° or at 20° C., while at 16° approximately 50 percent of the Turkey plants tillered from the coleoptile node, and at 12° the percentage increased to more than 80. Hard Federation, however, tillered rarely from this node, as the highest percentage of plants with coleoptile tillers was but four.

The number of seminal roots of Turkey was increased by germinating the seed at 25° C. as compared with 15°. Large seed and well-matured seed also increased the number of seminal roots. With deep seeding (75 mm) the number of seminal roots was much less than with shallow seeding (20 mm deep).

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# CROWN AND ROOT DEVELOPMENT IN WHEAT VARIETIES<sup>1</sup>

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## INTRODUCTION

Spring grains grown under extremely dry soil conditions in certain parts of the Columbia River Basin often do not form crown roots but grow to maturity from seminal roots alone. Even fall-sown wheat occasionally may mature with few or no crown roots if it is sown shallow or if it emerges late in the fall and the following spring is dry. When the crown roots fail to develop, yields are reduced, and the plants break over at maturity just below the soil surface. Observations of the varieties grown at the Sherman Branch Experiment Station, Moro, Oreg., have indicated that there is an apparent varietal variation in elongation of the subcrown internode that determines the depth of the tillering nodes from which the crown roots develop. This suggests a possible correlation between the depth of the tillering nodes or crown and hardness in wheat varieties, and the possibility that varieties may differ in the rate and ability to form crown roots under certain cultural and environmental conditions.

This paper presents the results from studies on some of the factors determining crown and root development, based on the examination of approximately 125,000 plants. The studies were started without the knowledge that somewhat similar investigations had been made by Tavčar (14)<sup>2</sup> and Friedberg (4) in Germany.

## REVIEW OF LITERATURE

Taylor and McCall (15) have presented a review of the literature on effect of environmental factors on morphology of the wheat seedling. From experiments under controlled conditions they show that the subcrown internode and coleoptile elongate more when wheat is grown at higher temperatures than when it is grown at temperatures of 16° C. or lower. They also show that in deeper planting the crowns form deeper in the soil.

Weaver (16) found that crown-root development in wheat plants began with the appearance of tillers. Janssen (7) states that in winter wheat all new roots in the spring develop from the crown of the plant and not from old roots.

Worzella (17), in a recent investigation on root development, found that the nonhardy varieties of wheat had a tendency to root horizontally, whereas the hardy varieties rooted obliquely or straight downward.

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<sup>2</sup> Reference is made by number (italic) to Literature Cited, p. 583.

Many investigators have stated or implied that the seminal roots are only temporary. Others, however, notably Krassovsky (8) and Cravzov (2), contend that the temporary root system of cereals lives and functions until the death of the plant. Krassovsky found that in wheat salt absorption continued in the seminal roots throughout the vegetative period, reaching its maximum near the time of heading, when the main stem attains its maximum growth. She found a relation between the seminal roots and the main stem and between the crown roots and the tillers.

The common opinion among many investigators, including Carleton (1), Robbins (13), Locke and Clark (9), and Rabotnowa (12), was that the crown-root system is formed from nodes at a rather constant depth below the soil surface no matter how deep the seed is sown. Robbins states that the crown roots are formed at a depth of 1 inch below the surface, and Locke and Clark state that the crown roots arise usually just below the surface of the soil.

Govorov (6), Tavčar (14), and Friedberg (4) found that the depth of the tillering node varied with the variety and that the deeper situated tillering node was associated with winter hardness. Dickson (3) and McKinney (11) found that the depth of the tillering node varied with different environmental factors, such as depth of seeding, amount of light, and temperature.

Friedberg (4) found that the tendency to produce tillers from the coleoptile node, which is a varietal character, allows of recovery after the growing point has been destroyed by frost; this explains why some varieties, although susceptible to frost, recover better than other varieties of equal susceptibility that seldom develop tillers from the coleoptile.

Gladkii and Lykhvar (5) found that varieties differed in ability to develop tillers from the coleoptile node and that the extent of development of tillers from this node increased with injury to the crown tillers.

#### EXPERIMENTAL METHODS

Varieties of winter and spring wheat were seeded at Moro, Oreg., on several dates during the year in order to get germination under different light and temperature conditions. After crown or root formation, which took place from 2 to 4 weeks after emergence, the tops of the plants were cut even with the ground with a sharp knife or scissors, and the crowns and subcrown internodes of the plants were dug. The distance from the seed and crown of each plant to the surface of the soil was recorded in millimeters. The difference is the length of the subcrown internode.

The term "seminal roots" is used in this paper to include those roots which form at the seed. According to McCall (10), the roots that arise from the coleoptile node are not true seminal roots. However, under field conditions it is difficult to distinguish them from the true seminal roots, primordia of which are present in the embryo. Crown roots are those that form at nodes above the coleoptile node. The internode above the coleoptile node, which elongates to determine the position of the crown, is the subcrown internode. The crown is the series of nodes with short internodes that forms usually below the surface of the soil. A tiller may be formed from each node in the crown, the number of tillers thus being determined by environment and variety.

## EXPERIMENTAL DATA

## VARIETAL VARIATION IN DEPTH OF CROWN

In 1931 a study was begun to determine the variation in crown depth of the winter wheat varieties in the plot trials at Moro., Oreg. These varieties were sown with a grain drill on October 15, 1930, and emerged November 6. At least 50 plants were examined in the spring of 1931 from each of three locations on each of three plots, or a total of 450 plants of each variety.

The study was continued in 1932, when 350 plants of each of the same varieties were examined. All varieties were sown with a grain drill on October 28, 1931, but owing to dry soil and cold weather, they did not emerge until early in March 1932.

The average depth of crown and length of the subcrown internode for the 25 varieties are given in table 1. The maximum and the minimum depths of crown found on any plants in 1931 are also given.

The data in table 1 show that the varieties ranked in about the same order for depth of crown in the 2 years. The varieties ranked in nearly inverse order for average length of subcrown internode, indicating that the depth of seeding averaged nearly the same for all varieties. The maximum and minimum crown depths found in plants of the same variety in 1931 show that considerable variability may occur.

TABLE 1.—Depth of crown and length of subcrown internode of 25 varieties of winter wheat grown in the field at Moro, Oreg., in 1931 and 1932

Variety	C. I. no <sup>1</sup>	Depth of crown					Length of subcrown internode		
		1931 <sup>2</sup>			Average for 1932 <sup>3</sup>	2-year average	1931 <sup>2</sup>	1932 <sup>3</sup>	2-year average
		Maxi- mum	Mini- mum	Average					
		Mm	Mm	Mm	Mm	Mm	Mm	Mm	Mm
Rio.....	10061	90	38	51	46	48.5	12	13	12.5
Golden.....	10043	80	33	54	39	46.5	8	21	14.5
Oro.....	8220	80	30	49	43	46.0	11	17	14.0
Turkey selection.....	11425	76	25	47	41	44.0	13	21	17.0
Kanred.....	5146	75	27	46	39	42.5	14	20	17.0
Kharkof.....	8249	79	30	46	38	42.0	15	22	18.5
Turkey (local).....	4429	77	24	47	37	42.0	11	27	19.0
Imbler.....	10066	75	28	46	38	42.0	12	26	19.0
Triplet.....	5408	71	27	46	36	41.0	9	26	17.5
Turkey.....	1571	69	25	44	36	40.0	14	24	19.0
Turkey selection.....	11424	75	25	46	34	40.0	14	31	22.5
Kharkof.....	1442	71	25	41	36	38.5	16	24	20.0
Fortyfold selection 51.....	10064	70	23	41	36	38.5	21	20	20.5
Ridit.....	6703	78	18	40	36	38.0	21	25	23.0
Areo.....	8246	76	20	43	33	38.0	25	32	28.5
Rex.....	10065	60	20	40	34	37.0	20	32	26.0
Fortyfold selection 29.....	10062	65	28	41	33	37.0	20	31	25.5
P1068 X Preston.....	8244	70	21	36	36	36.0	23	24	23.5
Hybrid 128.....	4512	60	20	40	32	36.0	18	25	21.5
Arcadian X Hard Federation.....	11426	60	18	41	31	36.0	15	28	21.5
White Odessa.....	4655	65	17	37	32	34.5	20	28	24.0
Fortyfold X Federation.....	8247	65	20	38	31	34.5	28	26	27.0
Kanred X Marquis.....	11423	69	21	37	25	31.0	24	40	32.0
Fortyfold X Hard Federation.....	11422	60	18	36	26	31.0	20	43	31.5
Federation.....	4734	35	8	24	22	23.0	28	38	33.0

<sup>1</sup> C. I. refers to accession number of the Division of Cereal Crops and Diseases

<sup>2</sup> Average of 450 plants.

<sup>3</sup> Average of 350 plants.

Varieties of the Crimean or Turkey group, and Golden, a selection from Goldcoin, had the deepest crowns. Oro, Rio, and Golden formed crowns more than 45 mm, or nearly 2 inches, below the soil surface. Federation, the only spring variety sown, formed crowns only about 23 mm, or less than 1 inch, below the soil surface (fig. 1).

Twenty-one winter wheat varieties were sown in nursery rows on October 17, 1933. Approximately 200 plants from each variety were examined in the spring of 1934. Table 2 gives their average depth of crown and length of subcrown internode. The average depth of crown ranged from 53 mm in Yogo and Turkey selection (C. I. 10016) to 28 mm in Federation. In this experiment, as in those made previously, the more winter-hardy wheats of the Turkey type formed their crowns deeper than did the less hardy, soft white wheats.



FIGURE 1.—Variation in crown depth of two wheat varieties (A, Rio; B, Federation) sown on the same date and at the same depth. The plants were cut at the soil surface.

Observations were made on depth of crown of 350 plants of each of 12 spring wheat varieties sown in the field at Moro on March 11, 1931. Similar results were obtained from the same varieties sown March 29, 1932, when 200 plants of each variety were examined. Table 3 gives the data for these spring wheat varieties. The crowns were formed nearer the soil surface than in the winter wheat varieties, and the differences in crown depth between varieties were smaller, the greatest being only 5.5 mm. The varieties, however, held about the same relation in the 2 years, indicating that spring wheat varieties do differ in the depth at which they crown.

TABLE 2.—Depth of crown and length of subcrown internode of 21 varieties of winter wheat grown in nursery rows at Moro, Oreg., sown Oct 17, 1933

Variety	C I no	Depth of crown <sup>1</sup>	Length of sub-crown internode <sup>1</sup>	Variety	C I no	Depth of crown <sup>1</sup>	Length of sub-crown internode <sup>1</sup>
		Mm	Mm			Mm	Mm
Yogo.....	8033	53	9	Albit.....	8275	40	18
Turkey selection.....	10016	53	8	Triplet selection R4-29.....		39	23
Oro.....	8220	50	4	White Odessa.....	4655	39	21
Rio.....	10061	50	8	Hybrid 128.....	4512	38	19
Turkey selection.....	11376	47	9	Turkey × Florence N.....			
Minhardt.....	5149	47	12	No 932.....		38	20
Golden.....	10063	47	14	Fortyfold × Federation.....	8247	36	23
Kharkof.....	8249	46	13	Hard Federation ×			
Kanred.....	4166	45	12	Martin.....	11488	32	27
Turkey selection.....	1592	44	15	Fortyfold × Hard Federa-			
Ridit.....	6703	42	15	tion.....	11422	32	25
Rev.....	10065	41	20	Federation.....	4734	28	31

<sup>1</sup> Average of 200 plants

TABLE 3.—Depth of crown and length of subcrown internode of 12 varieties of spring wheat grown in the field at Moro, Oreg., in 1931 and 1932

Variety	C I no	Depth of crown			Length of subcrown internode		
		1931 <sup>1</sup>	1932 <sup>2</sup>	2-year average	1931 <sup>1</sup>	1932 <sup>2</sup>	2-year average
		Mm	Mm	Mm	Mm	Mm	Mm
Marquis.....	4158	31	27	29 0	22	31	26.5
Pacific Bluestem.....	4067	30	25	27.5	24	33	28.5
Baart × Federation.....	8254	28	25	26.5	24	33	28.5
Baart.....	1697	28	25	26.5	25	33	29.0
Onas.....	6221	27	24	25.5	26	35	30.5
Federation.....	4734	27	24	25.5	30	36	33.0
Hard Federation selection 82.....	11421	27	23	25.0	30	34	32.0
Hard Federation selection 79.....	11420	27	23	25.0	30	42	36.0
Hard Federation.....	4733	26	23	24.5	31	37	34.0
Hard Federation selection 71.....	8256	27	22	24.5	31	35	33.0
Hard Federation 31.....	8255	27	22	24.5	31	37	34.0
White Federation.....	4981	25	22	23.5	30	39	34.5

<sup>1</sup> Average of 350 plants<sup>2</sup> Average of 200 plants

## VARIETAL VARIATION IN SUBCROWN PARTS

The internode above the coleoptile node usually is the only one to elongate below the crown in wheat plants. Under some conditions, however, one or more internodes above this one may elongate, and roots and a tiller may develop from each node.

Five wheat varieties were sown in small flats on May 14 and July 1, 1931, at Moro. After crowns had formed, 200 plants of each were examined to determine (1) the number of plants with two or more internodes immediately above the coleoptile node that had elongated and (2) the average length of these internodes. The data obtained are recorded in table 4. In these seedlings some plants in all varieties elongated in two internodes, but only Golden produced many plants that elongated in three internodes.

TABLE 4.—Percentage of plants with 1, 2, or 3 elongated internodes above the coleoptile node, and the average length of each internode, for several wheat varieties grown in Oregon under various conditions

Item	C I no	Depth of seed	First elongated internode		Second elongated internode		Third elongated internode	
			Plants	Length	Plants	Length	Plants	Length
Grown in field <sup>1</sup>								
Moro, 1931 <sup>1</sup>		<i>Mm</i>	<i>Pct.</i>	<i>Mm</i>	<i>Pct.</i>	<i>Mm</i>	<i>Pct.</i>	<i>Mm</i>
Federation.....	4734	64	100	46	28	14	4	6
Baart.....	1697	67	100	51	26	15	5	3
Hard Federation.....	4733	66	100	54	28	12	1	4
Golden.....	10063	61	100	39	29	11	13	23
Rio.....	10061	66	100	41	6	15	1	14
Moro, 1933 <sup>2</sup>								
Federation.....	4734	92	100	75	74	11	3 11	3
Hybrid 128.....	4512	99	100	62	79	16	1	1
Rio.....	10061	98	100	47	27	15	1	1 1/2
Oro.....	8220	98	100	48	35	15	0	—
Corvallis, 1933 <sup>2</sup>								
Oro.....	8220	119	100	75	23	23	0	—
Golden.....	10063	113	100	69	64	23	10	2
Grown in greenhouse								
Corvallis, 1933 <sup>2</sup>								
Federation.....	4734	88	100	70	25	17	0	—
Oro.....	8220	102	100	75	56	22	3 2	4

<sup>1</sup> Seeded May 14 and July 1; 200 plants of each variety were examined.<sup>2</sup> About 100 plants of each variety were examined<sup>3</sup> 3 plants had 4 elongated internodes each.

Table 4 also gives data recorded in 1933 at Moro and Corvallis, Oreg., relative to elongation of second and third internodes above the coleoptile node.

The tendency of wheat plants to elongate in two or three internodes has been observed under various conditions, but the elongation occurs more frequently in plants seeded deep or under high temperatures.

In many varieties some plants were observed to form tillers from the axil of the coleoptile. This tendency seems to vary with varieties and with environmental conditions. Under certain conditions, as high as 50 percent of the plants in some varieties developed a tiller from the coleoptile node. It is possible for the coleoptile tiller to continue growth after the main crown is entirely removed. The development of the coleoptile tiller may be a distinct advantage to plants in recovering after the crown has been badly injured.

About 200 plants each of 21 winter wheat varieties grown in field plots at Moro were examined in the fall of 1934. The number of plants with coleoptile tillers developed was recorded, and the results are given in table 5. Plants with coleoptile tillers were more abundant in 1934 than in any previous year. The low temperatures and slow growth of the varieties apparently were favorable to the formation of coleoptile tillers, as was found by Taylor and McCall (15).

TABLE 5.—Percentage of plants with coleoptile tillers in 21 varieties of winter wheat grown from seed sown in field plots Oct. 6, 1934, at Moro, Oreg.

Variety	C. I no.	Percentage of plants with coleop- tile tillers <sup>1</sup>			Variety	C. I no.	Percentage of plants with coleop- tile tillers .		
		Nov. 23	Dec. 4	Aver- age			Nov. 23	Dec. 4	Aver- age
Turkey selection.....	11425	80	85	83	Hybrid 128.....	4512	52	60	56
Do.....	10016	68	81	75	Hybrid 128 × White				
Blackhull.....	6251	64	86	75	Odessa.....	11607	34	75	55
Rex.....	10065	64	84	74	Rio.....	10061	51	58	55
Marquis × Kanred.....	11423	60	77	69	Turkey selection.....	11424	45	61	53
Turkey (local).....	4429	53	75	64	Federation.....	4734	38	64	51
Ridit.....	6703	54	65	60	Oro.....	8220	43	56	50
Kharkof.....	8249	53	65	59	Cheyenne.....	8885	39	51	45
Turkey.....	1571	55	63	59	Fortyfold × Hybrid 128.....	11700	43	47	45
Kharkof.....	1442	45	69	57	Golden.....	10063	39	48	44
Triplet.....	5408	48	65	57	Fortyfold × Federation.....	8247	28	48	38

<sup>1</sup> Based on 100 plants examined in each variety on each date

## INFLUENCE OF DATE OF SEEDING ON DEPTH OF CROWN

Baart and Federation spring wheats were sown at 10- to 15-day intervals during the spring and summer of 1931. Each variety was seeded about 43 and 65 mm deep. The depths of the crowns for the various seedings are shown in table 6. The crowns formed much shallower during the summer, when temperatures were high, than during the spring or early fall, when lower temperatures prevailed.

Rio and Golden winter wheats and Hard Federation spring wheat were sown on November 2, 1930, and on several dates in 1931. As shown in table 6, each of the three varieties had comparatively shallow crowns except when sown on November 2, 1930, and March 14, 1931, when temperatures were low.

TABLE 6.—Depth of crown of wheat varieties sown at various depths on several dates, and average air temperatures from seeding to 1 week after emergence, at Moro, Oreg., 1930-31

Date of sowing	Average air tem- perature	Average depth of crown	Depth of crown of wheat varieties sown at indicated average depth						
			Winter varieties		Spring varieties				
			Rio (65 mm)	Golden (63 mm)	Hard Federation (64 mm)	Baart		Federation	
						43 mm	65 mm	42 mm	64 mm
1930	°F	Mm	Mm	Mm	Mm	Mm	Mm	Mm	Mm
Nov. 2	35	43	51	54	24				
1931									
Mar 14	44	130 and 41	53	49	22	25	32	23	39
Apr. 6	49	30				30	32	23	34
May 1	58	22				20	23	22	23
May 14	61	20				20	20	18	20
July 1	68	20	31	22	8				
Aug. 2	68	15				12	16	15	18
Aug. 5	68	18	25	18	12	13	14	15	17
Aug. 16	70	15 and 19	21	21	14	13	14	15	17
Sept. 4	58	19 and 22	23	26	18	18	18	17	21
Average			34	32	16	20	22	19	25

<sup>1</sup> Baart and Federation.<sup>2</sup> Rio, Golden, and Hard Federation.

In 1933 four wheat varieties were sown at approximately 15-day intervals from July 26 to November 28. During this period daily maximum and minimum soil and air temperatures were recorded. The soil temperatures were obtained from a thermometer with the bulb placed about 2 inches below the soil surface. Shallow and deep seedings were made of each variety on each date. Table 7 shows the data obtained, including the depth of crown and seed for each variety under both shallow and deep sowing, and the average soil and air temperatures from date of seeding to 1 week after emergence.

TABLE 7.—Depth of crown of four wheat varieties sown shallow and deep on several dates during the summer and fall of 1933, and the average mean air and soil temperatures from seeding to 1 week after emergence, at Moro, Oreg.

Date of seeding	Average temperature		Average depth of crown	Depth of crown and seed, in millimeters, of indicated wheat variety sown shallow and deep															
				Federation				Hybrid 128				Rio				Oro			
				Shallow		Deep		Shallow		Deep		Shallow		Deep		Shallow		Deep	
	Crown	Seed		Crown	Seed	Crown	Seed	Crown	Seed	Crown	Seed	Crown	Seed	Crown	Seed	Crown	Seed		
July 26	°F	°F.	Mm	13	48	17	92	17	46	22	87	20	39	27	88	22	39	29	89
Aug. 15	71	76	21	12	50	16	81	18	48	23	79	22	48	28	86	23	45	30	85
Aug. 31	77	84	22	15	37	21	85	21	40	25	82	27	42	31	83	29	45	35	87
Sept. 20	60	66	26	22	48	30	99	33	48	37	99	44	52	51	98	44	51	50	98
Nov. 14	54	56	39	26	53	31	105	35	56	41	101	46	56	52	87	43	52	50	78
Nov. 28	42	39	41	26	47	30	81	36	52	42	86	48	55	52	82	42	47	58	82
Average	37	39	42	19	47	24	91	27	48	32	89	35	49	40	87	34	47	42	87

As in previous years, the crowns of all varieties were much shallower when sown during the warm summer weather. The crowns were deeper, especially in the winter wheats, from the seedings made in the period from September 20 to November 28, when temperatures were lower. In the first few seedings, crown roots started about 3 to 4 weeks after seeding, whereas in the later seedings, a longer period was required before these roots started.

#### INFLUENCE OF DEPTH OF SEEDING ON DEPTH OF CROWN

Baart and Federation spring wheats were sown about 43 and 65 mm deep on April 12, 1931. The average air temperature from seeding to 1 week after emergence was 52° F. An examination of 250 plants of each variety from each depth of seeding showed that the crowns averaged 25 mm in the shallow seeding and 31 mm in the deep seeding.

Four varieties of wheat were sown at three depths in the field at Corvallis, Oreg., on March 21, 1933. The average depth of the crowns of 150 plants of each variety at each depth of seeding is given in table 8. The difference in crown depth between shallow-seeded and deep-seeded wheat is shown in figure 2.



TABLE 8.—Depth of seed and crown in four wheat varieties sown at three depths in the field at Corvallis, Oreg., Mar. 21, 1933

Depth of sowing	Average depth of crowns of 150 plants of—				
	Federation	Hybrid 128	Oro	Rio	Average
Shallow (39 mm).....	<i>Mm</i> 18	<i>Mm</i> 21	<i>Mm</i> 25	<i>Mm</i> 23	<i>Mm</i> 22
Medium (59 mm).....	24	29	29	28	27
Deep (108 mm).....	29	39	45	43	39
Average.....	24	30	33	31	-----



FIGURE 2.—Variation in crown depth between shallow (39 mm) seedlings of Oro wheat, shown on the left, and deep (90 mm) seedlings, shown on the right, which were sown on the same date. Because of earlier emergence, the shallow-seeded wheat (left) is further advanced in growth

In the study on the effect of date of seeding on crown depth, made at Moro in 1934, each variety was seeded at two depths, as already shown in table 7. In all cases the deep-seeded plants produced crowns deeper on an average than those seeded shallow. The difference ranged from 3 to 16 mm, and the average depth of crown for all shallow-seeded plants in this study was 28.8 mm and for those seeded deep 34.5 mm.

Correlation coefficients between depth of seeding and depth of crown for Federation and Rio grown at Moro in 1932, are presented in table 9. A significant positive correlation was found between depth of seeding and depth of crown, especially with Rio, which crowns much deeper than Federation.

TABLE 9.—Correlation coefficients between depth of seed and depth of crown for Federation and Rio, grown at Moro, Oreg., 1932

Variety	Depth of sowing	Plants	Correlation coefficient between depth of seed and depth of crown
		Number	
Federation.....	Shallow.....	109	0.307±0.088
Do.....	Deep.....	105	.459±.077
Rio.....	Shallow.....	53	.671±.050
Do.....	Deep.....	228	.520±.049

Additional correlation coefficients were computed on part of the material grown at Corvallis, Oreg., in 1933. The varieties were separated into two groups, i. e., those normally shallow-crowned



FIGURE 3.—Effect of rain during a dry spring upon the crown-root development of spring wheat (Baart) sown on March 9, 1934. Plants on the right were dug on April 21 and those at the left on April 26. A rain of 0.3 inch fell on April 24. Because of dry surface soil, there were no crown roots prior to the light rain, but 48 hours afterward well-developed crown roots had formed.

and those normally deep-crowned. The correlation coefficient between depth of seeding and depth of crown in 566 plants of the varieties that normally crown shallow was  $0.753 \pm 0.013$ , and in 505 plants of the varieties that normally crown deep, it was  $0.765 \pm 0.011$ . The data show that there was a close relationship between depth of seeding and the depth at which the crown was formed.

#### VARIETAL DIFFERENCES IN TIME OF CROWN-ROOT DEVELOPMENT

Because of dry surface soil, spring wheats often do not form crown roots at Moro, Oreg. (fig. 3). Under these dry conditions, a variety that begins growth of its crown roots sooner than another may have a distinct advantage.

In the spring of 1931, and again in 1932, 50 plants each of the 12 spring wheat varieties grown in the varietal experiment were examined on four dates, at 2- to 3-day intervals, to determine the percentage of plants on which crown roots were developing. The results, summarized in table 10, show that Federation and Onas, two high-yielding spring wheats at Moro, formed their crown roots somewhat earlier than did the other varieties in the trial.

TABLE 10.—Percentage of plants of 12 spring wheat varieties having well-developed crown roots early in May, when varieties were sown Mar. 11, 1931, and Mar. 29, 1932, at Moro, Oreg.

Variety	C. I. no.	Plants having well-developed crown roots on—			Variety	C. I. no.	Plants having well-developed crown roots on—		
		May 2 to 8, 1931 <sup>1</sup>	May 3 to 11, 1932 <sup>1</sup>	2-year aver- age			May 2 to 8, 1931 <sup>1</sup>	May 3 to 11, 1932 <sup>1</sup>	2-year aver- age
		Per- cent	Per- cent	Per- cent			Per- cent	Per- cent	Per- cent
Federation.....	4734	91	71	81	Baart × Federation.....	8254	57	44	51
Onas.....	6221	81	70	76	Baart.....	1697	58	42	50
Pacific Bluestem.....	4067	65	84	75	Hard Federation selec- tion 79.....	11420	49	47	48
White Federation.....	4981	59	64	62	Hard Federation.....	4733	42	50	46
Marquis.....	4158	64	48	56	Hard Federation selec- tion 71.....	8256	47	44	46
Hard Federation selec- tion 82.....	11421	59	50	55					
Hard Federation 31.....	8255	54	52	53					

<sup>1</sup> 200 plants of each variety.

In the spring of 1934 a more extensive study of this nature was made on 10 of the 12 spring wheat varieties already studied. On March 9 the varieties were seeded in field plots with a grain drill. Counts were made on four dates of the number of plants with crown roots. These counts are recorded in table 11. This experiment was carried on under environmental conditions somewhat different from those in 1931 and 1932 in that the surface soil was so dry that crown-root formation was slow until after a rain of 0.3 inch on April 24. Up to that time, Pacific Bluestem and White Federation produced more plants with crown roots than the other varieties, as is shown in table 11 under date of April 21. After the rain, crown-root formation was very rapid, and in the final examination of 600 plants Pacific Bluestem and Federation ranked first and second, as shown under date of April 26. The percentage of increase between the third and fourth dates is given in the last column and shows that the most rapid increase in crown-root formation after the rain was in Federation.

TABLE 11.—Percentage of plants of 10 spring wheat varieties having well-developed crown roots on Apr. 12, 16, 21, and 26, when varieties were sown on Mar. 9, 1934, at Moro, Oreg.

Variety	C. I. no.	Plants having well-developed crown roots on—					Increase between Apr. 21 and Apr. 26 <sup>3</sup>
		Apr. 12 <sup>1</sup>	Apr. 16 <sup>1</sup>	Apr. 21 <sup>2</sup>	Apr. 26 <sup>2</sup>	Average	
		Percent	Percent	Percent	Percent	Percent	Percent
Pacific Bluestem.....	4067	27	17	52	90	54	73
Federation.....	4734	5	22	34	89	38	162
Baart X Federation.....	8254	8	29	38	88	41	132
White Federation.....	4981	13	42	50	86	48	72
Onas.....	6221	13	32	42	84	43	100
Hard Federation selection 7L.....	8256	4	20	45	83	38	84
Baart.....	1697	4	20	43	82	37	91
Hard Federation.....	4733	11	35	43	82	43	91
Hard Federation 31.....	8255	5	28	32	81	37	153
Marquis.....	4158	6	18	35	71	33	103

<sup>1</sup> Approximately 250 plants of each variety<sup>2</sup> Approximately 600 plants of each variety<sup>3</sup> Rain occurred on Apr. 24

In the fall of 1934, 200 plants each of the 21 winter wheat varieties grown in the varietal experiment at Moro, Oreg., were examined on two dates to determine the percentage of plants with crown roots developing. The results are given in table 12. It was found, as would be expected, that the rapid-growing, less hardy types of wheat formed their crown roots more rapidly than did the slow-growing Turkey types. This suggests a possible reason why in some localities certain soft winter wheat varieties that grow rapidly in their early stages and have a firmer root anchorage are better able to withstand injury from soil heaving than the slower growing, hardier Turkey types.

TABLE 12.—Percentage of plants of 21 wheat varieties having well-developed crown roots on Nov. 23 and Dec. 4, when varieties were sown on Oct. 6, 1934, at Moro, Oreg.

Variety	C. I. no.	Plants having well-developed crown roots on— <sup>1</sup>		Variety	C. I. no.	Plants having well-developed crown roots on— <sup>1</sup>	
		Nov. 23	Dec. 4			Nov. 23	Dec. 4
		Percent	Percent			Percent	Percent
Fortyfold X Federation.....	8247	79	87	Turkey selection.....	10016	2	14
Federation.....	4734	62	77	Marquis X Kanred.....	11423	2	7
Fortyfold X Hybrid 128.....	11700	35	69	Turkey selection.....	11425	0	4
Tripler.....	5408	41	64	Do.....	11424	2	3
Golden.....	10063	58	61	Turkey (local).....	4429	0	3
Hybrid 128.....	4512	19	60	Kharkof.....	1442	0	2
Rex.....	10065	31	58	Do.....	8249	2	1
Blackhull.....	6251	11	48	Turkey.....	1571	1	1
Ridit.....	6703	16	38	Oro.....	8220	1	1
Hybrid 128 X White				Rio.....	10061	0	1
Odessa.....	11607	17	35	Cheyenne.....	8885	0	1

<sup>1</sup> Based on 200 plants of each variety on each date.

Further observations will be necessary before definite conclusions can be drawn as to the extent and importance of varietal variation in time of crown-root development, but the observations already

made indicate that the time after emergence at which these roots form and the rapidity of their growth are varietal characteristics.

#### DISCUSSION

The data presented in this paper and the results obtained by other investigators justify the conclusion that depth of crown is a varietal character in wheat. This character is greatly influenced, and varietal differences are sometimes obscured, by environmental conditions, especially soil temperature. No data were obtained on the influence of light on crown-root development, because no adequate light-control equipment was available. Light intensity may be a factor in influencing the position of the crown in a wheat plant, as indicated by Tavčar (14), Friedberg (4), and others, but from the results reported in this paper it is believed that soil temperature is a much more potent factor.

The depth of the crown and the character of the crown-root growth in the early stages of growth of a wheat variety may have considerable effect on the extent of winter injury. Wheat varieties cannot definitely be classified for hardiness, however, on crowning depth. Many factors are involved in winter hardiness, and depth of crown is only one. In two varieties otherwise equal in hardiness, the one with the deeper crown would be better able to withstand low temperatures. It has been observed that differences in winter killing in the same variety sown deep and shallow may sometimes be quite marked, if the variety is one that normally crowns deeply. Better survival may be obtained from deep seeding, because of the better protection to the crown or growing point. Of the wheat varieties studied at Moro, Oreg., for crown depth, all of the deeper crowned varieties are of the hardy Turkey wheats except Golden, which is only a moderately hardy selection from Goldcoin. All of the less hardy varieties, especially those with a spring habit of growth, had comparatively shallow crowns. Depth of crown varied from 48 mm for Rio to 23 mm for Federation in one experiment and from 53 mm for Yogo and Turkey selection (C. I. 10016) to 28 mm for Federation in another.

Ability to withstand low temperatures and winter injury from soil heaving may not be closely associated. A variety only moderately resistant to low temperatures, with an early and vigorous root growth, is better able to withstand injury from soil heaving than a variety very resistant to low temperatures but with less vigorous root growth in its early stages.

Small differences were found in the crown depth of the spring wheat varieties studied, and differences were observed in the time and rapidity of crown-root growth. The latter may be of importance in connection with the ability of varieties sown in the spring to establish an adequate root system before the surface soil becomes so dry as to inhibit root growth. Federation forms its crown roots more rapidly than do some other spring wheats under normal conditions; under dry conditions that inhibit crown-root development, Pacific Bluestem and White Federation appear to be able to make most rapid root growth. When sown in the fall, Fortyfold  $\times$  Federation (C. I. 8247) and Federation form their crown roots much more rapidly than do the Turkey wheats.

The date of seeding affected the depth of crown of wheat varieties. Crowns were formed near the surface when seeds were sown in late spring or summer, when temperatures were high, and deep when seeds were sown in the fall or winter, when temperatures were low. When sown during cold weather, normally deep-crowned varieties may form crowns 45 to 50 mm below the surface, but when sown during warm weather they may have crowns only 20 to 25 mm deep.

The depth at which a wheat variety crowns may have an important bearing on the proper depth of seeding, a question of practical importance in the Columbia River Basin. The general recommendations in the past have been to seed winter wheat shallow, especially when the seeding is done late in the fall, when soil temperatures are low. Seeding too shallow, especially at early plantings, may result in more winter-killing. From the results of these studies it seems that seeding winter wheat much less than 38 mm deep would seldom be advisable. With favorable soil moisture and temperature conditions, winter wheat should be seeded from 51 to 64 mm deep, depending upon the variety. The best procedure would be to sow the seed at about the depth at which the crown would normally form, or slightly below. Deep seeding under normal conditions for winter wheat may produce crowns that will average 13 mm deeper than those produced by shallow seeding.

Depth of seeding influences the position of the crown, but the correlation is not high enough to warrant extremely deep seeding, even in spring wheat, in order to have deep crowns. Seeding spring wheat early and too deep will delay germination, emergence, and early root development. For late sowing, when soil temperatures are high, deeper seeding of spring wheat is advisable in order that the crowns may form as deep as possible.

#### SUMMARY

The depth below the surface of the soil at which the crown is formed in wheat plants is influenced by variety, environment (especially temperature), and depth of seeding.

Varieties differ greatly in the depth at which the crown is formed. Winter varieties form crowns deeper than spring varieties and, in general, hardy winter varieties form crowns deeper than nonhardy winter varieties.

Low temperatures between seeding and emergence cause the plants to crown deeper than do high temperatures.

Deeper seeding causes the crowns to be formed somewhat deeper but not in proportion to the difference in seeding depth.

Wheat varieties also differ in the time at which crown roots develop.

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# CERTAIN INSECT VECTORS OF *APLANOBACTER STEWARTI*<sup>1</sup>

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## INTRODUCTION

Bacterial wilt of corn (*Zea mays* L.) caused by *Aplanobacter stewarti* (E. F. Sm.) McC. was exceedingly destructive and more widely distributed during 1932 and 1933 than during any previous time in the history of the disease. Since 1897, when it was first described by Stewart, it has been studied by a number of investigators whose work has pointed more and more toward insects as a means of dissemination of the causal organism. Rand and Cash (?)<sup>3</sup> during 1920-23 found that bacterial wilt could be transmitted from diseased to healthy corn plants by two species of flea beetles, *Chaetocnema pulicaria* Melsh. and *C. denticulata* (Ill.), and by the spotted cucumber beetle, *Diabrotica duodecimpunctata* (Fab.). Ivanoff (5) reported transmission from diseased to healthy plants by the larval stage of the corn rootworm, *Diabrotica longicornis* (Say), as it attacked the roots of young seedling corn plants. He also reported that the bacteria of *A. stewarti* entered the corn plants through wounds made by white grubs, the larvae of *Phyllophaga* sp., feeding upon the roots in infested soil. A summary of this work, together with a brief review of the other literature on this disease, has recently appeared elsewhere (1).

The results of experiments by previous investigators on soil transmission of the causal organism indicate that transmission through the soil to uninjured roots of corn plants is exceedingly rare, if it ever occurs. Similar experiments by the writers have yielded only negative results. Therefore special attention has been given to insects as vectors of the organism, and the evidence of insect dissemination, obtained largely during 1934, is presented in this paper.

## MATERIALS AND METHODS

In order to determine the importance of insects in the overwintering and dissemination of *Aplanobacter stewarti*, insects commonly found on corn during the growing season were collected from as many different localities and host plants as facilities for their study would permit. Insect vectors of this bacterial organism were determined

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<sup>2</sup> The writers are indebted to Nancy H. Wheeler and J. W. Scrivener, of the Bureau of Entomology and Plant Quarantine, U. S. Department of Agriculture, for much valuable assistance with details of the experimental work reported in this paper; also to H. S. Barber of the same Bureau for his aid in identifying *Chaetocnema* spp. and to G. M. Smith, of the Bureau of Plant Industry, U. S. Department of Agriculture, for seed of inbred and hybrid corn used in the work.

<sup>3</sup> Reference is made by number (italic) to Literature Cited, p. 608

by transferring various species which had been confined to infected corn plants in various types of cages to healthy corn plants in cages. Feeding habits and relative choice of host plants of the flea beetles (*Chaetocnema* spp.) were studied by confining single individuals of these species to the plants for a definite period by means of spring-clip cages<sup>4</sup> (fig. 1).

In order to determine which species of insects and what proportion of these contained *Aplanobacter stewarti* under field conditions, many species of insects collected on or near infected corn plants were disinfected externally in a 4-percent solution of sodium hydroxide for 7 minutes and then rinsed thoroughly in a 0.1-percent solution of hydrochloric acid before being crushed in sterile beef peptone broth for

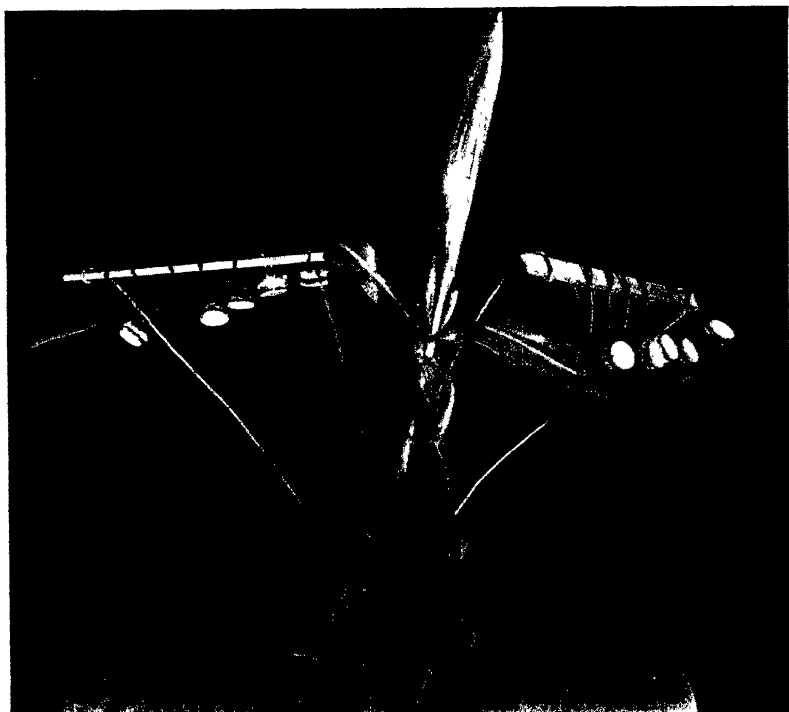


FIGURE 1.—Cages used to confine single individuals of *Chaetocnema* spp. and other insects to leaves of corn and other plants. These cages are often attached to broadleaved plants by means of the spring only, hence the term, "spring clip cages."

plating. The percentage of individuals of any species of insect carrying *A. stewarti* was determined by plating from individual insects, previously disinfected externally and crushed in sterile beef broth in separate glass vials, 9 by 34 mm. Usually more than 100 individuals were tested in each experiment. A holder for the vials was made by boring holes of the proper diameter and depth in a 1- by 4- by 12-inch board, in which the vials were placed in an upright position and numbered serially.

<sup>4</sup>These cages were furnished by the Division of Truck Crop and Garden Insect Investigations of the Bureau of Entomology and Plant Quarantine.



FIGURE 2.—*A*, Cultures of *Aplanobacter stewarti* isolated August 8, 1934, from *Chadocnema pulicaria*; colonies too small to be seen on this plate. *B*, Small section of the plate shown in *A*, magnified 10 times. Photographed August 14, 1934.

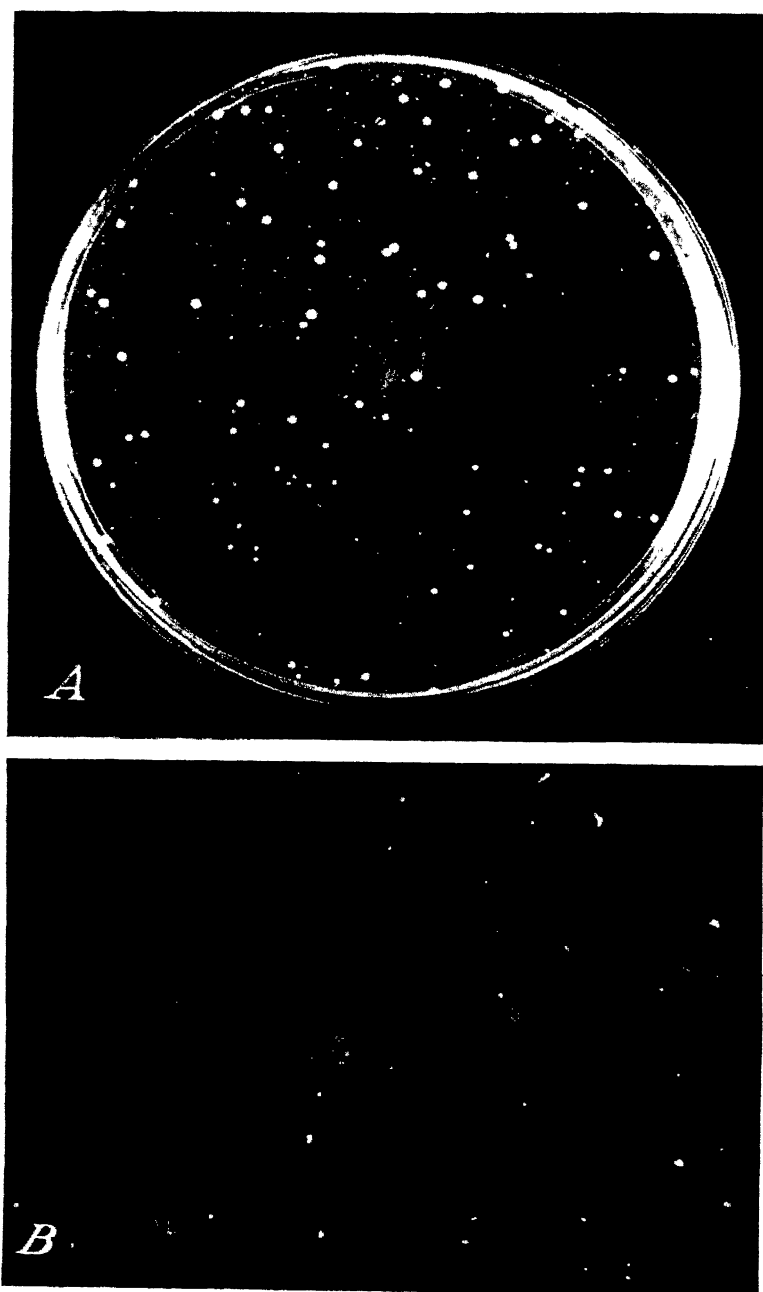


FIGURE 3—A, Dilution plate (made from culture poured in figure 2, A) showing individual colonies of *Aplanobacter stewarti*; B, Small section of the dilution plate shown in A, magnified 10 times. Photographed August 14, 1934.

Isolations of *Aplanobacter stewarti* from the broth cultures containing the crushed insect material were made by pouring plates in Difco beef peptone agar pH 6.7. The broth suspension was added to the first tube of agar and a dilution made from this. In most of the isolations of *A. stewarti* heavy seedings and practically pure cultures of the organism were obtained. On the first plate (figs. 2, A, and 3, A) the colonies were too abundant to be distinguished by the naked eye. On the second or dilution plate (figs. 2, B, and 3, B) separate colonies 1 to 3 mm in diameter developed. Transfers from individual colonies of all isolations were tested for pathogenicity by the inoculation of susceptible corn plants under controlled conditions in the greenhouse (fig. 4).



FIGURE 4—A portion of one bed in the greenhouse showing Golden Bantam sweet corn (Inbred 8482-3-1 from Glenn Smith) inoculated August 25, 1934, with isolations of *Aplanobacter stewarti* from *Chaetocnema pulicaria*. The large plants were inoculated but are free from the disease. Photographed September 11, 1934.

#### TESTS FOR TRANSMISSION BY INSECTS

At Arlington Experiment Farm, Rosslyn, Va., during 1933 and 1934, adults of *Chaetocnema pulicaria* (corn flea beetle), *C. denticulata* (toothed flea beetle), and *Diabrotica duodecimpunctata* (spotted cucumber beetle) were used successfully to transmit the bacterial wilt organism from diseased to healthy corn plants under controlled conditions. F. F. Dicke, of the Bureau of Entomology and Plant Quarantine, who was assigned to this project for a few months during 1933, obtained direct transmission of the disease from diseased to healthy corn plants by means of adults of the flea beetles *C. pulicaria* and *C. denticulata*. During January and February 1934 transmission from diseased to healthy plants was similarly effected by means of adults of the spotted cucumber beetle, *D. duodecimpunctata*.<sup>5</sup> The adult beetles were confined to corn plants, which were grown in 8-inch flowerpots, by means of cheesecloth-covered cages 2 by 2 feet by 2½ feet in height. A few attempts to transmit the disease from infected to healthy corn plants by means of the larvae of this beetle yielded

<sup>5</sup> These adults were kindly supplied by Charles E. Smith, in charge of a laboratory of the Bureau of Entomology and Plant Quarantine at Baton Rouge, La

only negative results. The bacterial wilt organism was isolated in pure culture from the plants infected by these beetles.

Other insects were tested in a similar manner to some extent during the summers of 1933 and 1934, and only negative results were obtained. The adults of these species were confined to diseased corn plants for several days and were then transferred to healthy plants in various types of cages and under a wide variety of conditions. The list of species is as follows:

<i>Chaetocnema confinis</i> Crotch	Sweetpotato flea beetle (a. n. o.). <sup>6</sup>
<i>Empoasca fabae</i> (Harr.)	Potato leafhopper (a. n. o.).
<i>Lygus pratensis</i> (L.)	Tarnished plant bug (a. n. o.).
<i>Nabis fersu</i> (L.)	Nabid bug.
<i>Systema taeniata blanda</i> (Melsh.)	Pale-striped flea beetle (a. n. o.).
<i>Chlorotettix viridius</i> Van D.	Leafhopper.
<i>Ceratomegilla fuscilabris</i> (Muls.)	Spotted ladybird.
<i>Thyanta custator</i> (Fab.)	Pentatomidae (stinkbug family).
<i>Phlepsius irroratus</i> (Say)	Irrorate leafhopper.
<i>Melanoplus differentialis</i> (Thos.)	Differential grasshopper (a. n. o.).
<i>Cicadula divisa</i> (Chl.)	Six-spotted leafhopper (a. n. o.).
<i>Heliothrips femoralis</i> Reut.	Thrips.
<i>Halticus citri</i> (Ashm.)	Garden flea hopper (a. n. o.).

The species of insects which have given positive results in the transmission of the wilt organism from diseased to healthy corn plants seem to be unequally endowed with the ability to induce infection. In the light of information now available it seems that *Chaetocnema pulicaria*, *C. denticulata*, and *Diabrotica duodecimpunctata* transmit the disease with facility in the order named. Many more lesions developed from feeding injuries of *C. pulicaria* than from those of the other two beetles. *C. pulicaria* also occurred in much greater abundance on corn throughout the season at Arlington Experiment Farm than either of the other two species.

#### OVERWINTERING OF APLANOBACTER STEWARTI

During April 1934 the beetles *Chaetocnema pulicaria* and *C. denticulata*, which had hibernated as adults, were collected from orchard grass (*Dactylis glomerata* L.), rye (*Secale cereale* L.), and alfalfa (*Medicago sativa* L.) at Arlington Experiment Farm and tested (as described under Materials and Methods) for the overwintering of *Aplanobacter stewarti*. A brief report on these studies has already been published (2). Comparatively few *C. denticulata* were available for this purpose and no wilt organisms were obtained from this species. *C. pulicaria* was fairly abundant, and, at first, collections of 100 or more were crushed together in one lot of broth. After large numbers of colonies of *A. stewarti* had been secured in practically pure culture from four such collections of these insects, 175 *C. pulicaria* collected from several different species of host plants were crushed individually, each in about 10 cc of the broth, in order to determine approximately what percentage of these beetles harbored the organism over winter. These tests indicated that approximately 19 percent of the beetles contained the organism in abundance. In the case of 100 *C. pulicaria* collected from alfalfa on April 30, one isolation was made from the abdomens and another from the heads and thoraxes. Heavy seedings

<sup>6</sup> Americano nomina officinale, i. e., common name accepted by the American Association of Economic Entomologists.

of *A. stewarti* were obtained from both. On May 1, 1934, two isolations were made from each of six adults of *C. pulicaria* collected from orchard grass, one isolation from the abdomen and one from the head and thorax. A heavy seeding of *A. stewarti* was obtained from the abdomen of one of these six flea beetles and a lighter seeding from the head and thorax of the same beetle.

As a further proof of the overwintering of *Aplanobacter stewarti* within the corn flea beetle, healthy corn plants in the greenhouse were infested for several days with other adult beetles from the collections referred to. Typical symptoms of bacterial wilt developed in these plants (fig. 5), and *A. stewarti* was isolated from them in pure culture.



FIGURE 5.—Sweet-corn plant inoculated with *Aplanobacter stewarti* by confining overwintered adults of *Chaetocnema pulicaria* to it for a few days during April 1934.

#### INSECT VECTORS OF *APLANOBACTER STEWARTI* IN THE FIELD DURING THE GROWING SEASON OF CORN

Isolation of *Aplanobacter stewarti* from externally sterilized insects was continued throughout the summer and fall to determine what percentage of the *Chaetocnema* collected from different host plants and localities were infested and to learn whether other insects also were concerned in the transmission of this disease under favorable field conditions. The results of this work are summarized in table 1.

Most of the insects collected were taken from or near corn infected with the disease during the active growing season. This fact explains the small numbers used in the case of several of the species tested, as they were found only occasionally on corn. In the case of *Chaetocnema pulicaria* and *C. denticulata*, the figures in the table include all individuals of these species from field collections which were tested, and also those used in the studies on overwintering of the organism within the insects. In several instances collections of these species were made from field corn at some distance from any known source of the disease in order to compare the results obtained with those of collections from or near infected corn. In several cases single cultures

represented many insects, sometimes over 100, instead of only single individuals.

TABLE 1.—Summary of insects collected in the field and cultured for *Aplanobacter stewarti*, 1934

Scientific name	Common name	Total specimens	Localities	Species of hosts	Isolations made	Isolations tested by inoculations on corn	Isolations of <i>A. stewarti</i>
		Number	Number	Number	Number	Number	Number
<i>Chaetocnema pulicaria</i> Melsh.	Corn flea beetle (a n o)	4,790	29	12	3,360	1,330	916
<i>Chaetocnema denticulata</i> Ill.	Toothed flea beetle.	905	16	9	384	89	30
<i>Chaetocnema confinis</i> Crotch	Sweetpotato flea beetle (a n o)	173	9	6	32	7	2
<i>Diabrotica duodecimpunctata</i> (Fab.)	Spotted cucumber beetle (a n o)	178	3	5	20	7	0
<i>Diabrotica</i> larvae		3	1	1	1	0	0
<i>Epitrix cucumeris</i> Harr.	Potato flea beetle (a n o)	365	13	6	49	5	0
<i>Epitrix parvula</i> (Fab.)	Tobacco flea beetle (a n o)	12	2	1	8	1	0
Carabid	Ground beetle	1	1	1	1	1	0
<i>Dibolia borealis</i> Chev.	Leaf beetle	42	2	2	3	0	0
<i>Disonycha ranthomelana</i> (Dalm.)	Spinach flea beetle	1	1	1	1	0	0
<i>Disonycha glabrata</i> (Fab.)	Flea beetle	2	1	1	1	1	1
<i>Systena taeniata blanda</i> (Melsh.)	Pale-striped flea beetle (a n o)	2	1	1	1	0	0
<i>Phyllotreta vittata</i> (Fab.)	Striped flea beetle (a n o)	6	1	1	3	0	0
<i>Stilbus viduus</i> Casey	Shining flower beetle	22	1	1	3	1	0
<i>Stilbus apicalis</i> (Melsh.)	do.	7	1	1	2	1	1
<i>Phalacrus politus</i> Melsh.	Smut beetle (a n o)	19	1	1	3	2	0
<i>Collops quadrimaculatus</i> (Fab.)	Beetle	3	1	1	2	0	0
<i>Epitachna corrupta</i> Muls.	Mexican bean beetle (a n o)	1	1	1	1	0	0
<i>Hypera punctata</i> (Fab.)	Clover leaf weevil (a n o)	3	1	1	1	0	0
Eulgorid	Lantern fly	3	1	1	1	0	0
<i>Empoasca</i> spp.	Leafhopper	47	1	1	4	3	0
<i>Eutettix seminudus</i> (Say)	do.	2	1	1	2	0	0
<i>Deltocephalus flavicosta</i> Stal.	do.	1	1	1	1	0	0
<i>Euscelus bicolor</i> (Van D.)	do.	4	1	1	1	1	1
<i>Agallia constricta</i> Van D.	do.	11	1	1	2	0	0
<i>Agallia sanguinolenta</i> (Prov.)	Clover leafhopper	2	1	1	2	0	0
<i>Cicadula dorsa</i> (Uh.)	Six-spotted leafhopper (a n o)	40	1	1	2	1	0
<i>Polyamia inimica</i> (Say)	Inimical leafhopper	1	1	1	1	1	0
<i>Thamnotettix nigrifrons</i> (Forbes)	Black-faced leafhopper	132	1	1	5	2	1
<i>Draculacephala mollipes</i> (Say)	Tenderfoot leafhopper	5	1	1	1	0	0
<i>Illinoia solanifolia</i> (Ashm.)	Potato aphid (a n o)	155	2	1	55	5	1
<i>Anaphothrips obscurus</i> (Muller)	Grass thrips (a n o)	66	2	1	9	1	0
<i>Aeolothrips fasciatus</i> (L.)		50	1	1	1	0	0
<i>Anaphothrips obscurus</i> (Muller)		50	1	1	1	0	0
<i>Orius insidiosus</i> (Say)	Insidious flowerbug	230	1	1	5	2	0
<i>Geocoris punctipes</i> (Say)	Plant bug	3	1	1	2	0	0
<i>Lycus pratensis</i> (L.)	Tarnished plant bug (a n o)	50	1	1	2	1	0
<i>Adelphocoris rapidus</i> (Say)	Plant bug	1	1	1	1	0	0
<i>Nabis ferus</i> (L.)	Nabid bug	1	1	1	1	1	0
<i>Micranthus calra</i> (Say)	Tree hopper	1	1	1	1	0	0
<i>Spinidurus</i> sp.	Springtail	8	1	1	2	0	0
Total (40 different species)		7,338			3,977	1,464	953

All of the insects tested, except the aphids and one lot of thrips, were sterilized externally before being crushed in the broth for plating. Samples of material from the collections of the various species used for isolations were referred to taxonomic specialists in the Bureau of Entomology and Plant Quarantine for identification.<sup>7</sup>

As indicated in the last column of table 1, isolations of *Aplanobacter stewarti* were obtained under these conditions from the following eight species: *Chaetocnema pulicaria*, *C. denticulata*, *C. confinis*,

<sup>7</sup> This assistance is hereby gratefully acknowledged



*Disonycha glabrata*, *Stilbus apicalis*, *Euscelis bicolor*, *Thamnotettix nigrifrons*, and *Illinoia solanifolii*. From all of these, except the *Chaetocnema* spp., *A. stewarti* was obtained in only a single instance. However, only small numbers of each of these species were taken directly from infected corn. Additional work is therefore necessary to determine the importance of these species as vectors of this organism. The effective isolation of *A. stewarti* from *I. solanifolii* was obtained from a lot of 48 individuals collected from infected sweet corn at Burlington, N. J., on July 17.

It is possible that the positive results obtained with *Chaetocnema confinis* may be due to mistaken identification, because the external characters given for distinguishing this species from *C. pulicaria* are probably not entirely reliable, and the whole genus appears to be greatly in need of a taxonomic revision. It is also very difficult to identify specimens of these species in a living condition because of their extreme activity. In some cases these insects were placed on a small cube of ice under the binocular microscope in order to separate the species from each other in a living condition. This method was not always satisfactory because in hot weather a film of moisture soon appeared on the thoraxes of the specimens and prevented a clear examination of the punctation there.

TABLE 2.—Percentage of *Chaetocnema pulicaria* infested with *Aplanobacter stewarti* at Arlington Experiment Farm, 1934

Date collected	Host plant	Adults cultured	Proportion yielding wilt organism	Date collected	Host plant	Adults cultured	Proportion yielding wilt organism
		Number	Percent			Number	Percent
May 1	Orchard grass.....	18	11 1	Aug 1	Sudan grass.....	100	19 0
14	Sweet corn.....	85	0	8	do.....	66	12 1
16	Orchard grass.....	4	25 0	10	Sweet corn.....	90	50 0
16	Wheat.....	57	17 5	10	Johnson grass.....	10	20 0
16	Sweet corn.....	54	18 5	15	Alfalfa and grass.....	100	9 0
18	do.....	83	13 2	19	Sweet corn.....	100	36 0
June 18	Johnson grass.....	100	0	28	do.....	100	47 0
20	Field corn.....	50	44 0	Sept 5	do.....	100	58 0
20	Sweet corn.....	50	40 0	11	Sudan grass.....	100	6 0
July 9	do.....	100	75 0	19	Sweet corn.....	45	56 5
12	Bindweed.....	17	5 8	19	Sudan grass.....	47	2 1
31	Sweet corn.....	100	38 0				

As reported in table 1, 4,790 specimens of *Chaetocnema pulicaria* were collected from 12 different host plants and from 29 localities during 1934. Of the 3,360 isolations made from this species, 916 yielded colonies of *Aplanobacter stewarti*, and transfers from these colonies produced typical wilt symptoms on young corn plants in the greenhouse. The host plants from which these *C. pulicaria* were collected were sweet corn, field corn, black bindweed (*Polygonum convolvulus* L.), alfalfa, young wheat (*Triticum aestivum* L.), Sudan grass (*Sorghum vulgare sudanense* (Piper) Hitchc.), Johnson grass (*Sorghum halepense* (L.) Pers.), tomato (*Lycopersicon esculentum* Mill.), oats (*Avena sativa* L.), cowpea (*Vigna sinensis* (L.) Endl.), yellow bristlegrass (*Setaria lutescens* (Weigel) F. T. Hubb.), sedge grass (*Cyperus* sp.), and weeds. The total of 908 adults of *C. pulicaria* collected from sweet corn at Arlington Experiment Farm during the

period May 14 to September 19 yielded an average of 40.3 percent infested with the wilt organism. Of the 669 adults of *C. pulicaria* collected from various other host plants (including field corn) during the same period at Arlington Experiment Farm, 12.1 percent were infested with *A. stewarti*. The results of tests with *C. pulicaria* collected at Arlington Experiment Farm during 1934 are presented in table 2.

The maximum proportion of *Chaetocnema pulicaria* from field collections found to contain *Aplanobacter stewarti* was 75 percent. These beetles were collected from infected sweet corn July 9.

Collections of *Chaetocnema pulicaria* from various localities have shown varying percentages of beetles which yielded the wilt organism when cultured in the usual manner. The results of these studies are given in table 3.

TABLE 3.—Percentage of *Chaetocnema pulicaria* from various localities infested with *Aplanobacter stewarti*, 1934

Date collected	Host plant	Locality	Adults tested	Proportion yielding wilt organism
			Number	Percent
May 5	Winter oats	Society Hill, S. C.	112	0 0
7	Field corn	Charleston, S. C.	145	0
11	Oats	Norge, Va.	59	0
June 6	do	Rising Sun, Md.	67	16 4
6	Field corn	do	50	6.0
6	Sweet corn	Princeton, N. J.	8	.0
6	Tomato	do	72	0
10	Oats	Sussex, N. J.	3	0
22	Field corn	Falls Church, Va.	50	28 0
22	Sweet corn	Annandale, Va.	50	16 0
27	do	Greenville, Pa.	16	25 0
27	Field corn	do	5	0 0
July 2	do	Falls Church, Va.	50	16 0
11	do	Charlottesville, Va.	54	55 5
11	do	Smithfield, Va.	50	6 0
17	Sweet corn	Burlington, N. J.	85	21.1
17	Field corn	Glasgow, Del.	97	19.5
18	Corn	Milford, Conn.	4	0
21	Field corn	Hamburg, Pa.	68	41 1
20	do	Kingston, N. Y.	5	.0
21	do	Gettysburg, Pa.	100	15 0
21	do	Harrisburg, Pa.	100	2 0
Aug 21	do	Franklin, Va.	100	40 0
21	do	Weldon, N. C.	27	18.6
30	do	Falls Church, Va.	100	29 0
Sept. 6	do	do	100	16.0

#### NUMBER OF INFESTED BEETLES LATE IN THE FALL

During the period of October 2 to November 22, several tests with beetles, involving 684 adults of *Chaetocnema pulicaria*, 39 of *C. denticulata*, and 48 of *Diabrotica duodecimpunctata*, were made in an effort to determine the percentage of these beetles that contained the bacterial wilt organism late in the fall of 1934, when they were about to hibernate. It was not always possible to obtain material for this work from areas immediately adjoining fields that had contained much of the disease during the previous summer. No *Aplanobacter stewarti* was isolated from *C. denticulata* and *D. duodecimpunctata*. Of the 684 adults of *C. pulicaria* tested, 90, or 13.1 percent, of the cultures yielded positive results. This probably was representative of the number of adult beetles of this species harboring

*A. stewarti* at this time. The collections of *C. pulicaria* used for these tests and the percentages known to harbor *A. stewarti* were as follows: Of 317 from Arlington Experiment Farm, 8.5 percent yielded *A. stewarti*; of 160 from Atchison County, Kans., 4.3 percent; of 110 from Platte County, Mo., 16.3 percent; and of 97 from Madison County, Ill., 39.1 percent.

#### HOST PLANTS OF APLANOBACTER STEWARTI

Until recently the only known host of the bacterial wilt organism, *Aplanobacter stewarti*, was the corn plant. This organism attacks



FIGURE 6—Golden Bantam sweet corn leaves showing three stages in the development of bacterial wilt lesions developing from insect feeding injuries. *A*, Feeding injuries and beginning lesions; *B*, feeding injuries and young but well-developed lesions; *C*, old lesions.

several kinds of corn, but the most susceptible host is sweet corn (fig. 6). During September 1934 natural infection with *A. stewarti* was found on teosinte (*Euchlaena mexicana* Schrad.), under field conditions at Lanham, Md., and at Arlington Experiment Farm, Va. Infected plants were from seed from Florida and Guatemala. The

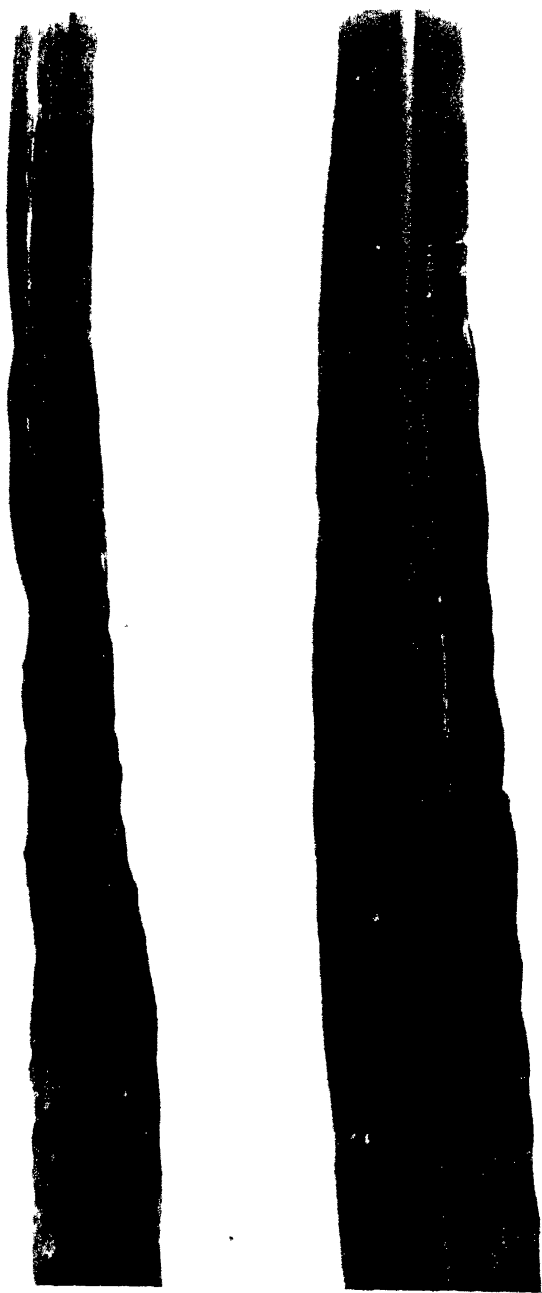


FIGURE 7—Natural infection with *Aplanobacter stewarti* on leaves of teosinte (*Euchlaena mexicana*) grown at Lanham, Md., from Florida seed. Photographed September 6, 1934.

lesions were similar to the leaf lesions on corn, but more limited than those occurring on susceptible varieties of corn. Isolations from these leaf lesions gave pure cultures of the typical yellow bacterium that produced wilt symptoms on Golden Bantam sweet corn in the greenhouse. A culture of *A. stewarti* isolated from the flea beetle *Chaetocnema pulicaria* produced long wilt lesions on teosinte leaves in the greenhouse and killed the plant. Adults of *C. pulicaria*, transferred from diseased corn to teosinte, produced wilt infection (fig 7), and the wilt organism was reisolated in pure culture.

Attempts to inoculate other plants, some of which are closely related to corn botanically, with pure cultures of *A. stewarti* by means of needle scratches and punctures, as well as by the feeding of adults of *C. pulicaria* which previously had been confined to diseased corn for a period of 5 days or longer, resulted in a failure to produce symptoms of the disease in any of these plants. The following species of plants were used in these studies: Goosegrass (*Eleusine indica* (L.) Gaertn.), smooth crabgrass (*Digitaria ischaemum* (Schreb.) Muhl.), eastern gamagrass (*Tripsacum dactyloides* (L.) L.), straw-colored cyperus (*Cyperus strigosus* L.), two varieties of sugarcane (*Saccharum officinarum* L.), Rimpan rye (*Secale cereale* L.), wheat (*Triticum aestivum*), Sudan grass (*Sorghum vulgare sudanense*), Johnson grass (*Sorghum halepense*), orchard grass (*Dactylis glomerata* L.), Kentucky bluegrass (*Poa pratensis* L.), and yellow bristlegrass (*Setaria lutescens*). More recent needle inoculations with cultures of *A. stewarti* have produced typical bacterial wilt lesions in

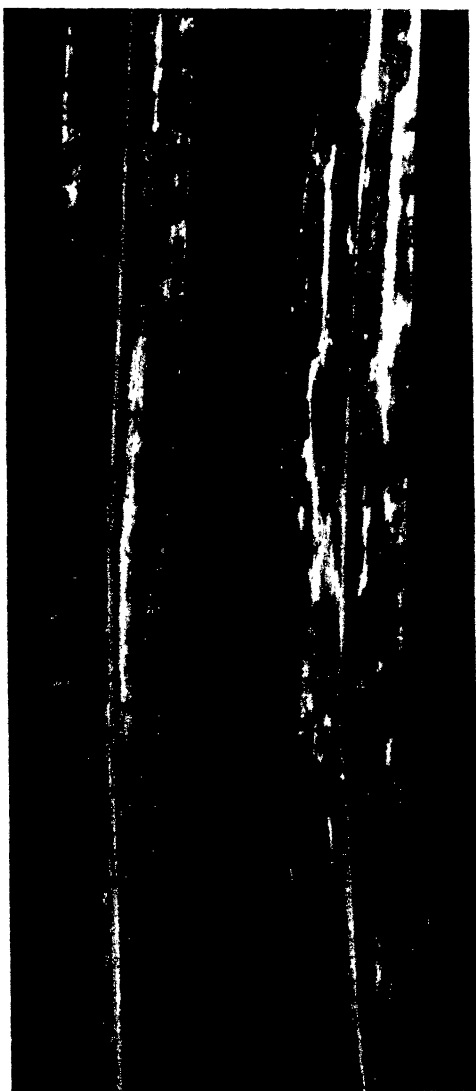


FIGURE 8.—Leaves of jobs-tears (*Cair* sp) showing lesions of bacterial wilt inoculated by infective adults of *Chaetocnema pulicaria* in confinement

the leaves of jobs-tears (*Coix* sp.), and the wilt organism has been reisolated in pure culture from this plant. Jobs-tears has also been inoculated with *A. stewarti* (fig. 8) by means of adults of *C. pulicaria* which had been transferred from infected corn to this plant.

#### HABITS OF CHAETOCNEMA SPP.

##### CHAETOCNEMA PULICARIA MELSH.

*Chaetocnema pulicaria* was reported as early as 1891 by Forbes (3, p. vii) as severely injuring corn in Illinois, and the injury described on corn strongly suggests the presence of bacterial wilt, which was unknown at that time. This insect has attacked corn in injurious numbers frequently enough to be known commonly as the corn flea beetle. It hibernates in the adult stage and becomes active on warm days early in spring when the air temperature reaches approximately 70° F. at the surface of the soil. In 1933 adults of this species were first collected at Arlington Experiment Farm on April 2, and in 1934 on March 21. The adults attack the young corn as soon as it appears above the surface of the soil. The immature forms of this species probably develop in the soil, although they have not been definitely

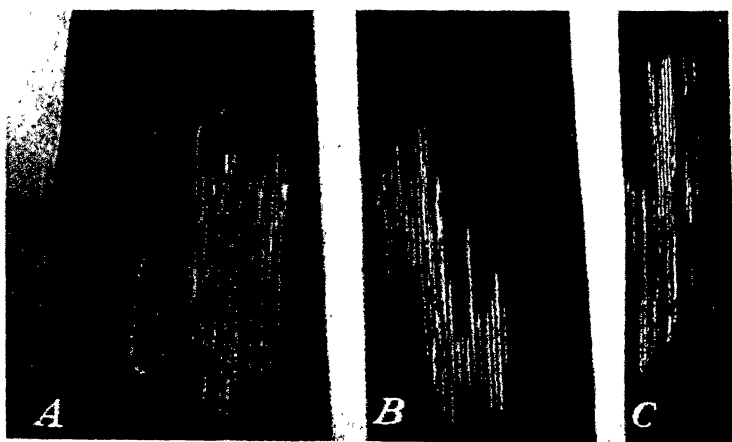


FIGURE 9.—Feeding by one adult of *Chaetocnema pulicaria* for 24 hours on sweet corn (A), Johnson grass (B), and *Cyperus* sp. (C).

identified in nature. The species is very small, the adults being 1.5 to 1.8 mm in length. They live for many months, are very active, and feed upon a wide variety of plants. Some adults have lived in the greenhouse at 65° F. from October to April. A few specimens passed through their life cycle on bluegrass in the greenhouse during the winter of 1934-35.

This species has been reported from most of the United States except the Northwest. Attempts to verify records of the reported occurrence in California have thus far been unsuccessful. Reports of the disease in California, therefore, suggest an insect vector other than those named in this paper, possibly the desert corn flea beetle, *Chaetocnema ectypa* Horn. Unpublished records on collections of insects made by airplane at Tallulah, La., by P. A. Glick during 1928-31 and made

available by him through the Division of Cotton Insect Investigations, Bureau of Entomology and Plant Quarantine, revealed the presence of *C. pulicaria* at altitudes ranging from 20 to 5,000 feet. This species was collected by airplane during nearly every month throughout the year at Tallulah. These data indicate that the species probably migrates or is carried in air currents for considerable distances. In two trap lights operated at Arlington Experiment Farm from May 6 to June 22 and from September 12 to December 5, 1934, one adult of *C. pulicaria* was collected November 3. The extreme activity of this insect was evidenced by the difficulty experienced in confining adults within cheesecloth cages even when a very fine mesh cloth was used. Field cages covered with similar cloth and used to exclude insects

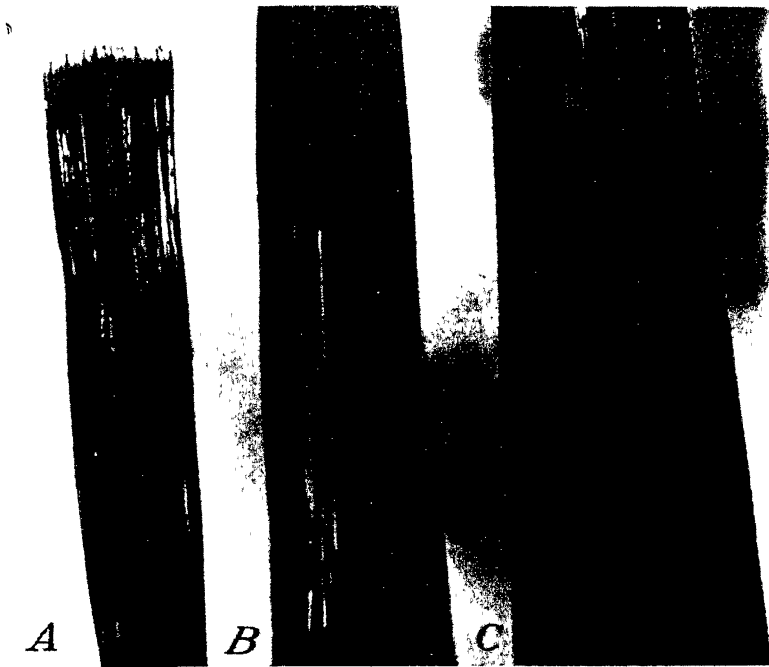


FIGURE 10.—Feeding by adults of *Chaetocnema pulicaria* on rye (A), teosinte (*Euchlaena mexicana*) (B), and eastern gamagrass (*Tripsacum dactyloides*) (C).

from hills of corn did not exclude this species unless the tacks holding the cloth were supplemented by strips of molding. From a collection of 50 adults of *C. pulicaria* taken from such cages on August 7, 34 (or 68 percent) yielded *Aplanobacter stewarti*, while a collection of 66 adults taken from Sudan grass at some distance away in the same field on the same afternoon yielded *A. stewarti* in only 8 instances.

The feeding habits of this species were studied on several host plants, and the amount and type of this feeding on the leaves of six species of plants are illustrated in figures 9 and 10. When the beetles were confined to corn in small cages, there was no apparent choice between feeding on the upper or on the lower surface of the leaves. In the field most of the feeding appeared to be on the upper surface. In

feeding, the adult always assumed a transverse position on the leaf and moved sidewise as it fed. It eats through to the epidermis on the opposite side and leaves a more or less transparent injury running parallel to the veins and appearing as a short, narrow, comparatively straight, white line.

Forty preliminary tests were made to determine the number of hours of feeding required for an adult of *Chaetocnema pulicaria* to inoculate healthy corn with the bacterial wilt organism, after it had fed at least 5 days on infected corn. Only negative results were obtained. These tests were carried on in the greenhouse in October 1934. Individual adults of *C. pulicaria* were confined to the leaves of corn plants in spring-clip cages (fig. 1) as follows: 10 for 2 hours, 10 for 6 hours, 10 for 8 hours, and 10 for 24 hours. Feeding apparently occurred in a normal manner and the plants grew slowly but normally; however, no lesions of the disease appeared on these plants.

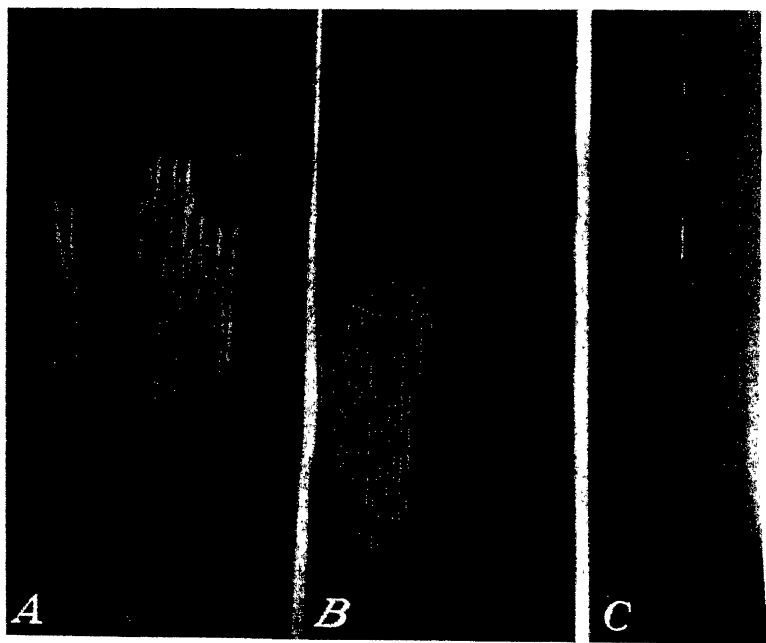


FIGURE 11.—Feeding by one adult of *Chaetocnema denticulata* for 24 hours on sweet corn (A), Johnson grass (B), and *Cyperus* sp. (C).

#### CHAETOCNEMA DENTICULATA (ILL.)

The adults of *Chaetocnema denticulata* are larger than those of *C. pulicaria*, ranging from 2.3 to 2.8 mm in length. Under field conditions at Arlington Experiment Farm during 1934 the species passed through at least two complete generations on Johnson grass. For the most part it was not so abundant as was *C. pulicaria* and fed much less on corn. The life cycle of a few individuals was completed under controlled conditions and extended over about 1 month. Under field conditions the eggs were found at the base of its host plant near the surface of the soil. The half-grown to full-grown



larvae are commonly found feeding on the tender unfurled leaves or on the developing seed head of Johnson grass. Pupation takes place in the soil, and the species overwinters in the adult stage. Feeding injuries on corn and Johnson grass are similar to those described for *C. pulicaria*, except that the green areas removed from the leaves are wider (fig. 11). The method of feeding appears to be identical with that of *C. pulicaria*.

#### CHAETOCNEMA CONFINIS CROTCH

*Chaetocnema confinis* has been reported as causing injury to corn similar to that caused by *C. pulicaria* (4, pp. 110-111). The present

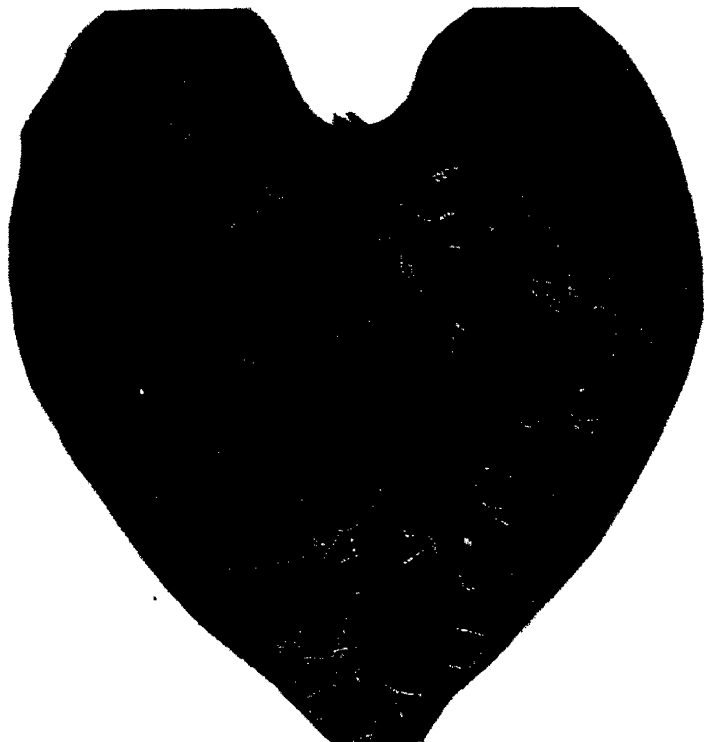


FIGURE 12.—Feeding injuries of *Chaetocnema confinis* to sweetpotato leaf. Note irregular paths of feeding.

writers' observations do not confirm this, although *C. confinis*, commonly known as the sweetpotato flea beetle, has often been collected on corn and Sudan grass when growing in proximity to convolvulaceous plants. A small number of feeding tests on corn under controlled conditions revealed no feeding injury in any way similar to that of *C. pulicaria* or *C. denticulata*. The adults of *C. confinis* appeared to die prematurely when confined to corn. On the other hand, the adults lived for long periods and fed considerably when confined to sweetpotato plants (fig. 12). This is an additional

reason for believing that the isolations of *Aplanobacter stewarti* obtained from *C. confinis*, as reported in table 1, may be due to misidentifications of the closely related species used.

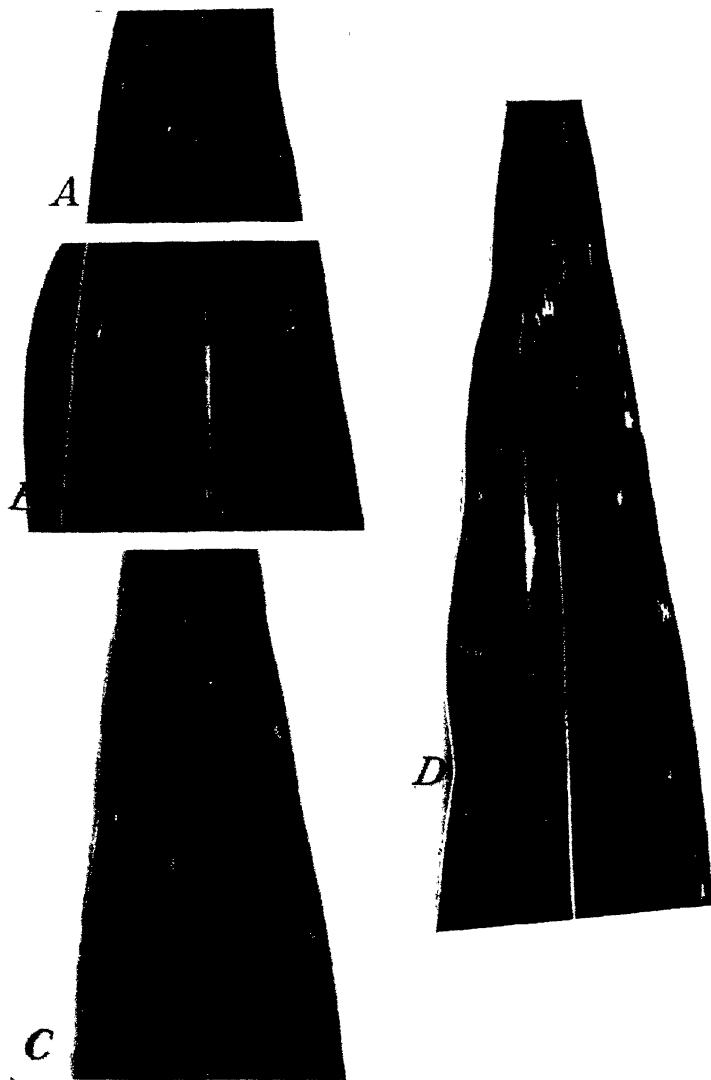


FIGURE 13 — Four stages in the development of a typical lesion of bacterial wilt following a small feeding injury to a sweet-corn leaf by *Chaetocnema pulicaria*, under field conditions. Photographed. A, August 1; B, August 6; C, August 10; D, August 13.

#### DEVELOPMENT OF LESIONS ON CORN

On August 17, 1933, a number of lesions of bacterial wilt, which started on the leaves of the corn at feeding places of flea beetles,

were measured to obtain definite data on the development of these secondary infections under field conditions on Golden Bantam corn grown from seed obtained from several localities. The lesions were marked with india ink, and each leaf was identified by means of a linen tag. Unusual, almost continuous, rainfall on August 21, 22, and 23 (5.88 inches on Aug. 23) interfered with making the measurements as planned. The final measurements were made August 26, and the results, presented in table 4, indicate the rapid development of the lesions.

TABLE 4.—Development of lesions of *Aplanobacter stewarti* following feeding by *Chaetocnema pulicaria* on leaves of Golden Bantam corn at Arlington Experiment Farm, 1933

Lot no	Source of seed	Lesions measured	Aug 17		Aug 26		Average increase in length
			Average length	Average width	Average length	Average width	
		Number	Mm	Mm	Mm	Mm	Percent
1.....	Oregon.....	17	16 8	2 7	248 2	10.7	1,377 4
2.....	Idaho.....	9	21 1	2 7	313 3	11 0	1,384 8
3.....	Seed company in District of Columbia.....	7	12.4	2 8	138 5	6 2	1,016 9
4.....	Do.....	18	11 9	2 0	160 5	6 4	1,248.7

It will be noted from table 4 that the average increase in length of lesions in four lots during a 9-day period varied from approximately 10 to 14 times the original size.

During 1934 a few similar measurements were made of lesions on leaves of a susceptible variety of corn, and four photographs were made of what appeared to be a typical lesion as it developed in the field (fig. 13). The measurements of this lesion are given in table 5.

TABLE 5.—Measurements of a lesion of *Aplanobacter stewarti* on a leaf of sweet corn, 1934

Date <sup>1</sup>	Length	Width	Date	Length	Width
	Mm	Mm		Mm	Mm
Aug. 1.....	7	1	Aug. 8.....	44	4
Aug. 3.....	10	2	Aug. 10.....	115	4
Aug. 6.....	38	4	Aug. 13.....	160	5

<sup>1</sup> The lesion was photographed on Aug. 1, 6, 10, and 13. On Aug. 17 the lesion coalesced with others.

#### MAXIMUM INFESTATION OF CHAETOCNEMA PULICARIA AND C. DENTICULATA BY APLANOBACTER STEWARTI

Tests were made to determine the percentage of adults of *Chaetocnema pulicaria* from which *Aplanobacter stewarti* could be isolated after being confined to infected corn in cages in the outdoor insectary for a minimum of 5 days. These insects were sterilized externally. The results are shown in table 6.

TABLE 6.—Percentage of *Chaetocnema pulicaria* containing *Aplanobacter stewarti* after confinement on infected corn for 5 days, 1934

Date cultured	Adults tested individually	Isolations of <i>A. stewarti</i>	Proportion containing <i>A. stewarti</i>
	Number	Number	Percent
Aug. 8.....	50	34	68.0
Aug. 23.....	75	23	30.7
Sept. 18.....	102	69	67.6
Sept. 28.....	115	64	55.7
Total.....	342	190	.....
Average (weighted).....	.....	.....	55.6

It will be noted from the table that only from 30.7 to 68 percent, or an average of 55.6 percent, of the adults of *Chaetocnema pulicaria* yielded *Aplanobacter stewarti* in these tests, even after these beetles had been confined to infected corn in cages for a minimum of 5 days. Another lot of 78 adults of *C. pulicaria*, tested at the same time by placing the surface-treated uncrushed insects on agar plates, yielded no colonies of *A. stewarti*. Of most interest in these latter tests was the fact that some of these beetles survived the entire process. Of 23 adults of *C. denticulata* confined to infected corn in cages for a minimum of 5 days, 11, or 47.8 percent, yielded colonies of *A. stewarti*. These percentages of beetles containing the wilt organism are unexpectedly low when compared with a maximum of 75 out of 100 adults of *C. pulicaria* that were found to contain *A. stewarti* when collected from plots of infected sweet corn under field conditions July 9, 1934, at Arlington Experiment Farm.

#### RETENTION OF APLANOBACTER STEWARTI BY THE BEETLES

Two series of experiments were conducted with *Chaetocnema pulicaria* and *C. denticulata* in order to determine how long adults of these beetles remained infective after being confined in cages for 5 days or more with diseased plants. The plants placed in the test cages were replaced by other healthy plants at intervals of not more than 5 days in order to prevent the possibility of the beetles becoming reinfected from the plants which they had inoculated. This possibility was considered very remote because of the slow development of the wilt symptoms under the conditions of these experiments. In both series of experiments the beetles of each lot apparently remained infective for approximately the entire period of their life.

In tests conducted in 1933 it was noted that some specimens of *Chaetocnema pulicaria* remained infective for a period of 22 days, i. e., they were able to inoculate susceptible sweet corn with the organism 22 days after having fed on infected corn. This observation suggested the tests made with overwintered adults in April 1934 which led to the discovery that *Aplanobacter stewarti* overwinters in the bodies of these flea beetles.

Tests made during 1934 indicated that one lot of 25 adults of *Chaetocnema denticulata* inoculated healthy corn plants with *Aplanobacter stewarti* from September 14 to October 20 inclusive (all of these adults died by Nov. 14), or for a period of at least 37 days.

In similar tests with a lot of 25 adults of *C. pulicaria*, healthy corn plants were inoculated from September 26 to November 13 inclusive (all of these adults died by Nov. 26), or for a period of at least 49 days. Similar tests made with adults of *Diabrotica duodecimpunctata* during February 1934 indicated that the adults of this species were able to transmit the bacterial wilt organism from diseased to healthy corn plants after being removed from the diseased plants from 5 to 9 days. Externally sterilized adults of this species which, after feeding several days on infected corn plants, had fed on healthy plants 6 and 15 days, respectively, did not yield the wilt organism when crushed in sterile beef peptone broth and plated in the usual manner.

#### BACTERIAL WILT AND ITS INSECT VECTORS IN RELATION TO WINTER TEMPERATURES

The association of warm winters with outbreaks of bacterial wilt has frequently been commented upon. Stevens<sup>8</sup> concluded that when the average winter temperatures are high, bacterial wilt has shown a tendency to increase, and vice versa. He reported that no obvious relation appears between the incidence of the disease and the total rainfall during any season, nor between the incidence of the disease and the temperature during spring, summer, or fall, although he stated that one may well exist. He established temperature indices for the winter months (sum of the mean temperatures of December, January, and February) and according to the data presented suggested that the disease will usually be absent in the North-eastern States following a winter with an index below 90° and present in destructive amounts following a winter with an index above 100°. The unusual abundance and distribution of this disease from 1929 to 1933 followed a series of mild winters. The amount of bacterial wilt in 1934 was much reduced following the low temperatures of the previous winter. This was particularly true in eastern New York and in New England, where the known insect vectors were almost entirely absent during 1934. The relationship of winter temperatures to the abundance and distribution of the insects which overwinter and transmit *Aplanobacter stewarti* may be very significant and has received some attention. *Chaetocnema pulicaria* was not found abundantly north of central Pennsylvania during 1934. *A. stewarti* was isolated from this insect from all localities where this beetle was found abundantly.

The effect of the extensive use of resistant varieties of sweet corn on the amount of bacterial wilt is a factor that should not be overlooked in predicting abundance of this disease. The problem is doubtless further complicated by lack of information on the abundance of the insect vectors in which the organism of the disease overwinters. The relative abundance and infectivity of the hibernating insect vectors may be of extreme importance in predicting the appearance of bacterial wilt of corn in destructive abundance.

#### DISCUSSION

Since *Aplanobacter stewarti* has been known to overwinter in seed only to a limited extent (2 to 13 percent) and there is no direct

<sup>8</sup> STEVENS, N. E. STEWART'S DISEASE IN RELATION TO WINTER TEMPERATURES. U. S. Dept. Agr., Bur. Plant Indus. Plant Disease Repts 18 141-149, illus. 1934 [Mimeographed]

evidence of its overwintering in naturally infested soil in the field, the finding of this organism overwintering in a virulent condition in the corn flea beetle, under natural conditions, offers a satisfactory explanation as to the source of early spring infections. Furthermore, the high percentages of beetles infested with the organism throughout the corn-growing season readily account for the midseason spread of the disease.

The problem of determining the part played by insects in the overwintering and dissemination of bacterial wilt of corn is a very large one. Neiswander (6), in his paper on the sources of American corn insects, reports that 352 species of insects have been recorded in the literature as attacking the corn plant. He suggests that some of these records are probably obsolete and have never been verified by later observations. However, he lists 166 species as significant corn insects. How many of these may be carriers of the wilt organism remains to be determined. Isolations for *Aplanobacter stewarti* were made during 1934 from 40 species of insects belonging to 33 genera. Eight species representing six genera yielded *A. stewarti*. Only the corn flea beetle (*Chaetocnema pulicaria*) and the toothed flea beetle (*C. denticulata*) appeared to be of importance in the dissemination of the disease. Much further work is necessary in order to determine whether the other six species involved are normally vectors of *A. stewarti*.

Transmission of bacterial wilt from infected to healthy corn was effected by means of adults of the spotted cucumber beetle (*Diabrotica duodecimpunctata*), but none of the 178 adults of this species collected throughout the season under field conditions yielded *Aplanobacter stewarti*.

Collections of *Chaetocnema pulicaria* from or near plantings of sweet corn at Arlington Experiment Farm at intervals of 7 to 14 days yielded an increasing number of isolations of *Aplanobacter stewarti* up to July 9, on which date a maximum of 75 out of 100 individuals yielded this organism. After this date the number again decreased. The maximum proportion of *C. pulicaria* that yielded *A. stewarti* after being confined to infected corn in cages for at least 5 days was 68 percent, and the average of the 342 such beetles tested was 55.5 percent. It seemed that this percentage should have been higher under these circumstances. The isolations of *A. stewarti* obtained from *C. pulicaria* collected from sweet corn at Arlington Experiment Farm during the period May 14 to September 19 averaged 40.3 percent, whereas similar collections from other host plants (including field corn) during the same period yielded an average of 12.1 percent. In collections of *C. pulicaria* from various localities throughout the season of 1934, from 0 to 75 percent of the individuals in each collection yielded *A. stewarti*.

Very rapid development of lesions of bacterial wilt following feeding on the corn leaves by the corn flea beetle was observed under field conditions. The average increase in length of lesions in four lots during a 9-day period varied from approximately 10 to 14 times the original size. The increase in size of the wilt lesions was greater on the Golden Bantam corn grown from seed produced in Oregon and Idaho than on corn grown from seed produced in wilt-infected areas. Plants produced from seed grown in a section where the disease does not occur are often more susceptible to bacterial wilt than are plants from seed grown in wilt-infected areas.

## SUMMARY

Bacterial wilt of corn is not transmitted through the soil; this finding confirms the results obtained by earlier workers.

Direct transmission of bacterial wilt from infected to healthy corn was effected by means of three species of beetles, *Chaetocnema pulicaria*, *C. denticulata*, and *Diabrotica duodecimpunctata*, thus verifying earlier work of this type. Similar tests with 13 other species of insects yielded only negative results.

*Aplanobacter stewarti* was found to pass the winter in a virulent condition in the bodies of the hibernating adults of the corn flea beetle, *Chaetocnema pulicaria*. Approximately 19 percent of 175 of these overwintering beetles which were tested during April 1934 yielded *A. stewarti*. Young corn plants are inoculated with bacterial wilt in the spring by the feeding of these beetles, and this appears to be the chief source of early infection each year.

A total of 7,338 insects, representing 40 species in 33 genera, collected on or near corn infected with bacterial wilt, during the corn-growing season in 1934, were tested for the presence of *Aplanobacter stewarti*, and eight species yielded this organism. A total of 3,977 isolations were made, 1,464 of which were suspected of containing *A. stewarti* and were tested by inoculation on corn, 953 yielding positive results.

Of 908 adults of *Chaetocnema pulicaria* collected from sweet corn at Arlington Experiment Farm, during the period May 14 to September 19, an average of 40.3 percent yielded *Aplanobacter stewarti*, whereas of 669 adults of the same species collected during the same period from various other host plants (including field corn) at the same place an average of 12.1 percent gave *A. stewarti*. The maximum proportion of isolations of *A. stewarti* obtained from adults of *C. pulicaria* was 75 percent.

Collections of adults of *Chaetocnema pulicaria* from 12 different host plants and from 29 different localities yielded *Aplanobacter stewarti* in from 0 to 75 percent of the individuals in each collection.

Of the 684 adults of *Chaetocnema pulicaria* collected and tested late in the fall when they were about to hibernate, 13.1 percent yielded *Aplanobacter stewarti*.

In addition to corn, teosinte (*Euchlaena mexicana*) and jobs-tears (*Coix* sp.) were found to be hosts of *Aplanobacter stewarti*. Infection was obtained both by needle inoculations and through the feeding of infective flea beetles, *Chaetocnema pulicaria*. Tests with several other grasses yielded only negative results.

The length of bacterial wilt lesions, following feeding injury by adults of *Chaetocnema pulicaria* on corn in the field, was found to increase approximately 10 to 14 times the original size during a 9-day period in 1933.

Compared with 1932 and 1933, the amount of bacterial wilt in eastern New York and in New England was much reduced in 1934 following the low temperatures of the previous winter. *Chaetocnema pulicaria* was not found abundantly north of central Pennsylvania during 1934. The relationship of winter temperatures to the abundance and distribution of the insects which overwinter and disseminate *Aplanobacter stewarti* may be very significant in predicting the appearance of bacterial wilt of corn in destructive abundance.

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# GROWTH INHIBITION IN THE POTATO CAUSED BY A GAS EMANATING FROM APPLES<sup>1</sup>

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## INTRODUCTION

When the potato, *Solanum tuberosum* L., is confined with gases emanating from apple fruits its growth is arrested. This reaction was first noted in investigations of temperature effects on the pathogenicity of *Rhizoctonia* to the potato. All the potato seed pieces kept in incubators in a cold-storage room containing apples produced small, abnormal sprouts (?).<sup>3</sup> Subsequent investigations have proved that a volatile substance normally produced by ripe apple fruits was the cause of this growth inhibition.

The object of this paper is to describe the morphological and physiological effects on the potato of the growth-inhibiting gas from apples, and to present information concerning the characteristics and chemical identity of this substance.

Sound, nondormant potato tubers, growing potato plants, and ripe apples (fruits of *Malus malus*) were used in these studies. The potatoes, usually cut into seed pieces, were planted in moist soil, sawdust, or sphagnum, under conditions favorable for their growth. The planted seed pieces or the growing plants, together with a few apples, were placed in a closed container in order to provide a sufficient concentration of the growth-inhibitory gas from the apples, but the fruits were never in actual contact with the potatoes. As a rule, containers with a capacity of approximately 2 cubic feet or less were used, and generally the tests were made in the greenhouse. The controls consisted of potatoes growing under similar environmental conditions except that apples were omitted from the container.

## MORPHOLOGICAL CHANGES IN THE POTATO PLANT DUE TO THE PRESENCE OF APPLES

### EFFECT ON THE SPROUTS

The volatile substance from apple fruits affects the potato plant by markedly limiting the growth of the stem and roots. The limited sprout growth develops radially approximately as fast as longitudinally, and there results a tuber like structure closely appressed to the potato eye (fig. 1, *A*, and *B*). Radial enlargement is most rapid near the apex, where the tissues are still meristematic. Rupturing of the outer layers near the apical end frequently occurs (fig. 2, *B*).

Abnormally thickened and fleshy rootlets sometimes develop from the sprouting eyes (fig. 3, *C*). When normal potato roots are exposed to the gas from apples, apical growth ceases, and abnormal enlargement in diameter occurs near the tip (fig. 2, *B*).

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<sup>2</sup> The studies of the chemical nature and characteristics of the growth-inhibitory gas were performed with the advice of Dr. H. N. Barham, of the Kansas State College of Agriculture and Applied Science, Department of Chemistry.

<sup>3</sup> Reference is made by number (italic) to Literature Cited, p 626.

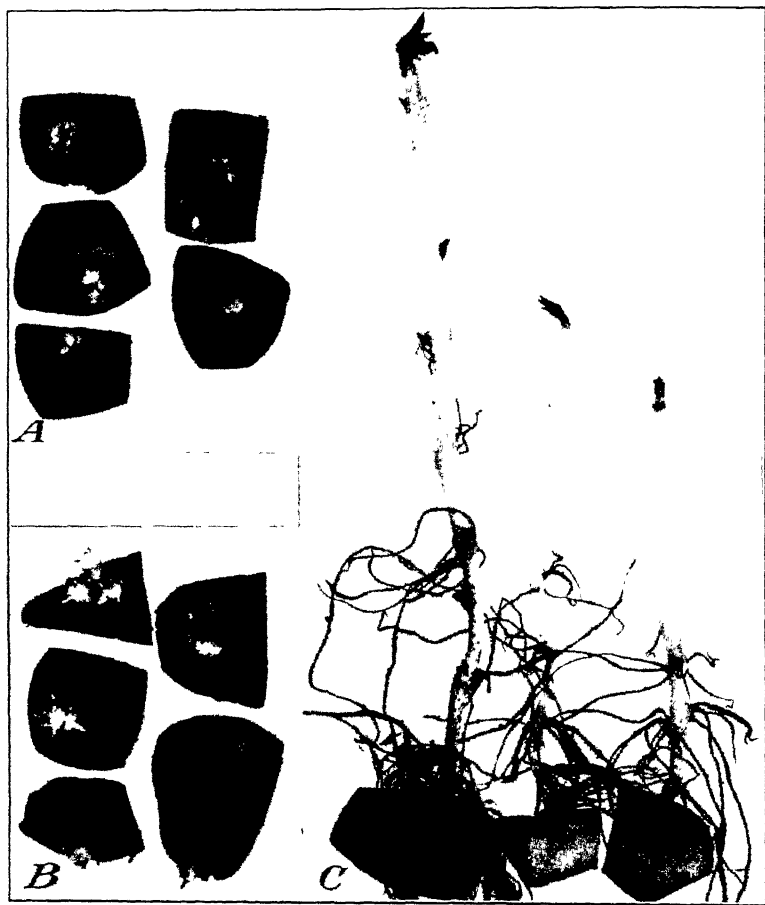


FIGURE 1.—Sprout development in potatoes exposed to growth-inhibiting gas: *A*, Stunted, abnormal sprouts from seed pieces confined 10 days with apples; *B*, stunted, abnormal sprouts from seed pieces confined 10 days with hawthorn fruits; *C*, normal sprouts from control seed pieces

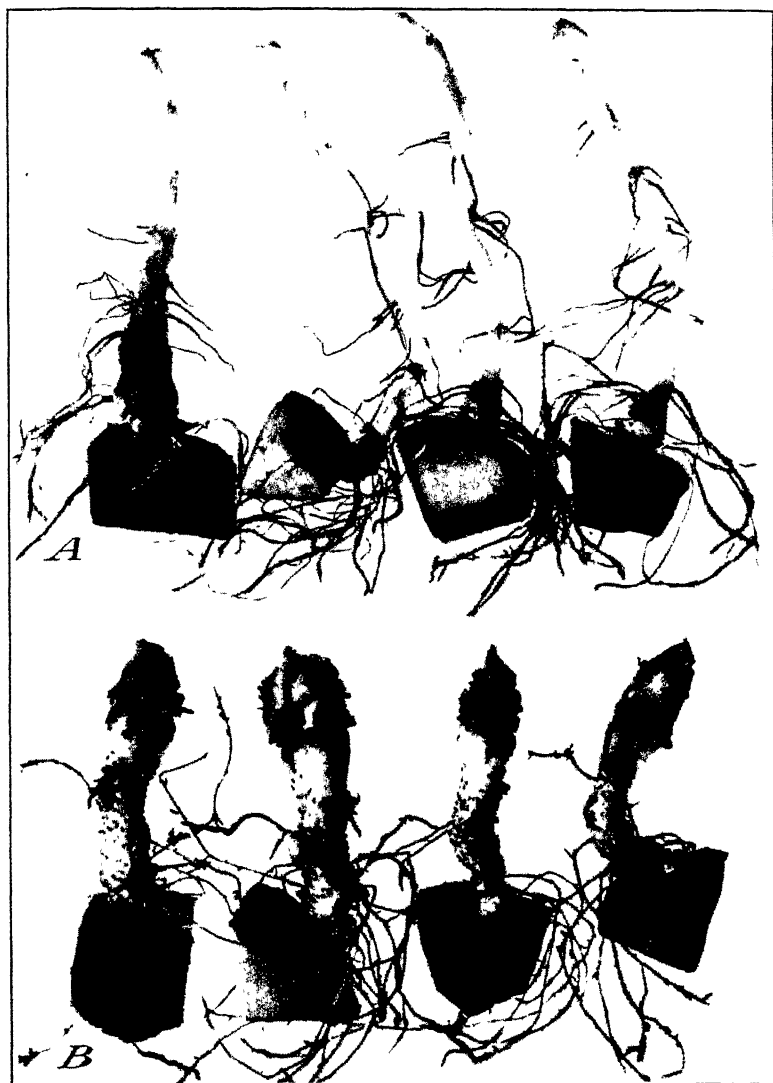


FIGURE 2.—Duration of the effect of growth-inhibiting gas on sprout development: *A*, Sprouts grown 7 days with apples and then 7 days without apples, *B*, sprouts grown 7 days without apples and then 7 days with apples.

A test was made early in these investigations to determine whether the conditions under which potatoes had previously been kept had any effect upon their response to the growth-inhibiting gas from apples and whether different varieties of potatoes reacted similarly when confined with apples. Tubers of Early Ohio and Irish Cobbler from Minnesota, Netted Gem from Idaho, Spaulding Rose No. 4



FIGURE 3.—A, Normal sprouts developed from tubers confined 14 days with cider, B, abnormal sprouts developed from tubers confined 14 days with pressed pulp of apple fruits; C, abnormal sprouts developed from tubers confined 14 days with sound apples, D, normal sprouts from the controls.

from Maine, Bliss Triumph from Montana, White Triumph from western Kansas, and an undetermined variety from Oregon were tested. All of these lots of potatoes produced the characteristic abnormal growth, and the responses of the different varieties were similar.

The gas from apples may suppress the bud dominance in the tuber eye and thus make it possible for buds to develop that would normally

remain dormant and for multiple sprouting to result. This was particularly noticeable in the Triumph and Early Ohio varieties, in which from 7 to 12 buds started growing from numerous single eyes. Single buds, however, also developed from some of the eyes of these same tubers, but the potatoes that were confined with apples produced a greater number of sprouts on an average than did the control tubers.

A test was made to determine whether the effect of apples on bud dominance persists after the tubers are planted in the field. Forty Irish Cobbler and 40 Bliss Triumph tubers that had been held in storage with apples were cut into seed pieces and then planted. They

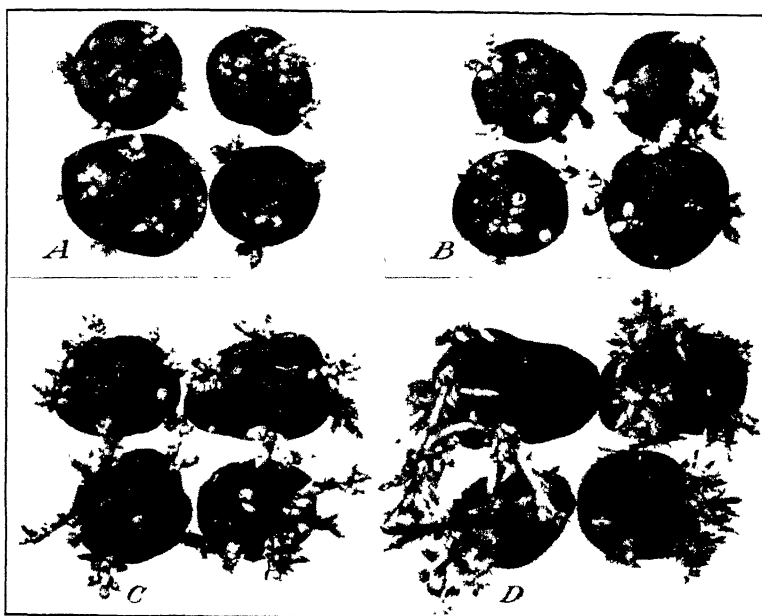


FIGURE 4.—Preservative effect of apples on 60-pound lots of potatoes stored with apples from January until June in 5-cubic-foot containers. *A*, Tubers stored with 10 pounds of apples; *B*, tubers stored with 5 pounds of apples; *C*, tubers stored with 1 pound of apples; *D*, control tubers

produced 198 and 171 sprouts, respectively, while the 40 control tubers of each of these varieties produced 159 and 135 sprouts, respectively. In this test the Irish Cobbler and Bliss Triumph tubers that had been held with apples both produced approximately 25 per cent more sprouts than did the control tubers.

When tubers were planted in moist soil under favorable growing conditions and confined with apples, abnormal sprout development was more rapid than was the case when the tubers were kept dry. Figure 3, *C*, illustrates the sprouts that developed in 2 weeks from treated tubers planted in moist soil, and figure 4, *A*, illustrates the smaller sprouts produced by tubers held with apples from January until June in a commercial potato-storage house.

## DURATION OF THE EFFECT

The inhibition of normal growth apparently becomes operative as soon as potato plants are placed under the influence of a sufficient concentration of the growth-inhibiting gas. When affected potatoes are removed from its presence, normal growth is apparently resumed immediately.

Pots containing sprouting seed pieces planted in soil were placed in two closed cans, one of which contained apples. At the end of 7 days the seed pieces in the can containing apples had produced stunted sprouts, whereas those not exposed to apple gas had produced normal sprouts averaging more than 5 cm in length.

The two lots of seed pieces were interchanged; those that during the first 7 days of the test had produced normal sprouts were placed in the container with apples, and those grown in that container the first 7 days were transferred to the container in which there were no apples. During the next 7 days the seed pieces in the container with the apples made no appreciable growth in length, but their stems enlarged radially 300 percent (fig. 2, *B*). Radial enlargement was most rapid near the apex, where the tissues were still meristematic. Frequently the outer layers ruptured near the apical end.

Linear growth started immediately in the sprouts stunted the first 7 days of the test (fig. 2, *A*) and averaged more than 8 cm during the 7 days they were in the container without apples. The basal radial enlargements were still evident at the end of the second week.

Seed pieces which had been planted in soil and confined with apples for 2 weeks were planted at a depth of 3 inches in a soil bed, and a like number of seed pieces which for the preceding 2 weeks had been planted in soil but not confined with apples were planted alongside at the same depth. The short, thickened sprouts of the seed pieces confined with apples were not removed. Only sprouts that were 5 mm or less in length were allowed to remain on the seed pieces that had not been confined with apples. No significant difference was noted in the time required for sprout emergence from the two lots. This indicates that the effect of the growth-inhibiting substance did not persist after the potatoes were removed from this gas.

## EFFECT ON THE GREEN PLANT

Above-ground portions of the potato plant are definitely affected by the growth-inhibiting gas from apples. Normal growth ceases, the leaf blades, leaf petioles, and stems become abnormally rigid, and the older leaves bend downward as though wilted. Crocker (4) noted this effect, known as epinasty, on certain other plants when they were subjected to the gases ethylene, acetylene, propylene, butylene, and carbon monoxide.

The drooping of the potato petioles began within an hour after the plants were placed under a bell jar with apples, and the response was very noticeable after 2 or more hours. The time lag of an hour before visible symptoms appeared was probably due to the fact that the necessary dosage of the inhibitory gas had to accumulate and that it took the plant some time to react.

The potato plants were allowed to remain under bell jars for several days (fig. 5) in this experiment. After 3 or 4 days the older leaves of plants exposed to the apple gas became chlorotic. This was followed

by the loss of all green color, after which the petioles became detached by abscission. The very young leaves at the tip of the stem remained green and in an upright position but became rigid and did not grow perceptibly. While terminal growth of the plants held with apples was practically stopped, there occurred an abnormal radial growth or thickening of the stems and of the petioles of the partly developed leaves that remained attached. This thickening of the leaf petioles was greatest at the axils. Control plants held under bell jars without apples produced a normal growth.

#### EFFECT ON STORED POTATOES

Potatoes were stored with apples in boxes with a capacity of 5 cubic feet from January until early summer in order to obtain information on the effect of the gases from this fruit in preserving the tubers.



FIGURE 5.—Effect of the growth-inhibiting gas from apples on the potato plant. *A*, Control plant held 2 weeks under bell jar; *B*, potato plant confined 2 weeks with apples. The oldest leaves of *B* became chlorotic and died, the younger leaves curved downward, became rigid, and the petioles enlarged radially, especially at the axils. The stem also became thickened.

This experiment was first made in 1933 and was repeated in 1934. The storage boxes, which were not completely closed in order to allow ventilation, were held in a commercial potato-storage house at Topeka, Kans., which was kept at approximately 10° C. until March, after which the temperature rose because of the warmer weather, as artificial cooling facilities were not available.

Sixty pounds of sound Irish Cobbler potatoes were placed in each of four of the boxes on January 29, 1933, and to three of them were added respectively 1, 5, and 10 pounds of Winesap apples. The potatoes in a fourth box containing no apples served as a control. The test was completed June 24, at which time the four lots of potatoes were weighed, and final observations were made on their condition.

The tubers that were stored with the 5-pound and the 10-pound lots of apples developed the abnormal sprout growth that is induced by the gas from apples. They had remained firm, and none had decayed.

Inhibition of sprouting was most pronounced in the potatoes that were stored with 10 pounds of apples, and these tubers were in the best state of preservation, as is indicated in figure 4, *A*. The potatoes that were stored with 1 pound of apples produced sprouts that exhibited an unmistakable growth inhibition, although they resembled the controls closely. Only two potatoes had decayed, but the tubers were shriveled and in poor condition (fig. 4, *C*). The control tubers (fig. 4, *D*), on the other hand, had sprouted profusely, and were decidedly shriveled and in poor condition, and a few had decayed. The sprout growth from the control tubers was abnormally branched, and many of these sprouts, particularly in the lower layers of tubers, were dead. This condition was probably due to the lack of ventilation in the boxes. The loss in weight of the control tubers, after the sprouts were removed, was 8.75 pounds, and the loss in weight of the tubers that were held with 1, 5, and 10 pounds of apples was respectively 8.5, 7.25, and 4.75 pounds.

The potatoes that had been stored with 10 pounds of apples tasted sweeter than did the control tubers, indicating that more of the starch had been hydrolyzed to sugars. This effect is of considerable importance in regard to the practicability of using the growth-inhibiting gas from apples for preserving stored potatoes.

The storage test was repeated in 1934, but the quantities of apples in the three boxes were increased to 5, 10, and 20 pounds. The test was started in January and completed in June, and the results were similar to those obtained in 1933. The tubers that were confined with apples produced only a limited sprout growth, and they were better preserved than were the control tubers. A considerable number of tubers, however, were decayed, and comparative weight decreases which were due to respiration and evaporation could not be obtained. The state of preservation and the sprout development of the potatoes stored with the 10-pound and with the 20-pound lots of apples were similar. This suggests that under the conditions of these storage tests, 10 pounds of apples supplied to 60 pounds of potatoes a sufficient concentration of the growth-inhibiting gas for maximum prevention of sprouting.

In order to learn what effect the volatile gas from apples has on seed potatoes, tubers of the varieties Irish Cobbler and Bliss Triumph were planted in the field after they had been confined with apples for 60 days. Untreated tubers were planted as controls. The time required for the emergence of sprouts was similar for both the untreated and treated lots. The treated Cobbler and Triumph tubers produced respectively 25 and 27 percent more sprouts than did the control plants, but the yields were similar. The increased number of sprouts from the treated tubers is evidently an effect of the gas from apples, which causes bud dominance in the potato eye to be overcome. This test indicated that potatoes to be used for planting may be preserved with the growth-inhibiting gas from apples without harmful effects to their productivity.

#### SOURCE OF THE GROWTH-INHIBITING GAS

All the apple varieties that were tested caused growth inhibition of the potato, and this characteristic appears to be common to all ripe apples. Abnormal growth response of potato sprouts were obtained with Winesap, Stayman Winesap, Jonathan, Ben Davis, Gano, Delicious, Rome Beauty, and Yates apples.



Certain other fruits and vegetables were kept in closed containers with sprouting potatoes in order to ascertain whether, like the apple, they produce a growth-inhibiting gas. An inhibited sprout growth was produced by pears, *Pyrus communis* (varieties Bosc and Kieffer), and by the fruits of the hawthorn, *Crataegus crus-galli* (fig. 1, B). Normal growth resulted when the planted tubers were subjected to the gases produced by bananas, onions, oranges, or sweetpotatoes.

Immature apples did not cause growth inhibition in the potato plant, and it appears that the growth-inhibiting substance is not produced by such fruits. When the immature apples were held, however, until they had ripened and were again placed in an enclosed chamber with potatoes, they caused the characteristic growth inhibition.

It was noted that the growth-inhibiting substance is produced in smaller quantities by overripe fruits and not at all by decayed fruit tissue. When sound but overripe pear and hawthorn fruits were confined with potatoes, the resultant sprout growth was thickened, elongated slowly, and was more or less intermediate between the normal and the abnormal. A similar but less definite response was noted in overripe apples. When potatoes planted in soil were placed in a chamber with decaying apples, abnormal sprouting developed as long as healthy apple tissue was present, but normal growth began when the apples were entirely decayed.

Studies were made to determine what tissues in the apple produce the growth-inhibiting gas. Freshly isolated mesocarp, endocarp, skin, and seeds were renewed daily in closed chambers containing sprouting potatoes. Inhibited growth similar to that occurring when the whole fruit was used resulted in the potatoes that were confined with the mesocarp and with the endocarp tissues. Abnormal growth of sprouts did not occur on the potatoes held in containers with the skin or the seeds of apples; and it appears, therefore, that these portions of the apple do not produce the growth-inhibiting gas.

Growth inhibition was not obtained from potatoes that were confined with cider but did result when the potatoes were confined with pressed apple pulp. Ground apples were pressed in a hydraulic press under 19,000 pounds pressure, and the juice and pulp fractions were placed in separate closed chambers containing planted potatoes. Each portion of juice and pulp was allowed to remain in the container for a day and was then replaced by a newly prepared portion. The normal sprout growth from the potatoes confined with the cider and the abnormal growth from those that were held with the pulp is shown in figure 3.

Steam distillate from crushed apples was confined with sprouting potatoes but did not prevent normal sprout production. Newly prepared portions of the distillate were supplied daily, and each portion was allowed to remain but 1 day with the potatoes. The growth-inhibiting substance was evidently not concentrated in the distillate from the fruits.

## CHANGES IN NORMAL PHYSIOLOGICAL PROCESSES

### RESPIRATION

Measurements were made on the intensity of respiration from sprouting potato tubers that were growing in normal atmosphere and from tubers that were held in a similar environment with the excep-

tion that the atmosphere contained the volatile emanations from apples.

The two lots of tubers were each confined in an enclosed chamber through which a stream of air was constantly drawn. The air supplied to the control-tuber lot was obtained directly from the laboratory and permitted normal sprout growth. The air supplied to the other lot of potatoes was charged with the volatile gases from apples by drawing air from the laboratory through a cylinder 25 cm in diameter and 75 cm long, filled with ripe apples. These potatoes produced the characteristic abnormal sprouting that results when growing potatoes are confined with the growth-inhibiting gas from apples.

A measure of the intensity of respiration of the experimental potatoes was obtained from the increase in weight of soda lime after the salt had absorbed the carbon dioxide respired from weighed lots of the potatoes for measured periods of time. Atmospheric carbon dioxide was removed from the stream of air supplied to the growing chambers by first passing the air through soda lime. Calcium chloride was employed to dry the air before it entered the soda-lime towers.

Sound, uncut tubers were used, and their weight was recorded at the beginning of the test when they were placed in the enclosed growing chambers. Not less than 1,000-g samples of tubers were used. The different tests, which were made at laboratory temperatures of approximately 20° to 25° C., varied in length from 10.5 to 21 days. A moist atmosphere was provided for certain lots by packing the tubers in moistened sphagnum, while other lots were kept in a dry atmosphere.

Four series of tests on respiration intensity were made, and the results are recorded in table 1. This table records the weight increases of the soda lime from carbon dioxide respired by the experimental tubers. In order that comparisons may be readily seen, there is given in the table the computed weight of carbon dioxide respired from 1,000 g of tubers during a period of 21 days. The percentage increase of carbon dioxide respiration from the affected tubers in excess of that from the nonaffected tubers is also given.

TABLE 1.—*Effect of the growth-inhibiting gas from apples upon respiration from sprouting potato tubers*

Date	Potatoes held moist or dry	Growth-inhibiting gas absent				Growth-inhibiting gas present			Increase of carbon dioxide in abnormal potatoes
		Length of test	Weight of tubers	Carbon dioxide respired	Carbon dioxide respired per 1,000 g potatoes per 21 days	Weight of tubers	Carbon dioxide respired	Carbon dioxide respired per 1,000 g potatoes per 21 days	
		Hours	Grams	Grams	Grams	Grams	Grams	Grams	Percent
October 1932.....	Dry.....	504	1,191	12.79	10.74	1,247	28.64	23.77	92
	Moist.....	504	1,290	10.48	8.12	1,190	20.75	17.44	
January 1933.....	Dry.....	504	1,045	5.47	5.23	1,180	20.52	17.40	79
	Moist.....	504	1,045	6.29	6.02	1,205	14.07	11.68	
January 1934.....	do.....	253	1,000	2.46	4.91	1,050	17.15	16.33	150
	Dry.....	310	1,030	3.23	5.10	1,050	10.32	9.83	
February 1934.....	Dry.....	310	1,035	2.87	4.51	1,045	13.41	12.83	85
	Dry.....	310	1,035	2.87	4.51	1,050	9.88	9.41	
						1,000	5.29	10.54	115
						1,040	9.20	14.38	182
						1,030	9.14	14.43	220

## CATALASE ACTIVITY

Measurements were made on the catalase activity of potato tissue from tubers that had been affected by the growth-inhibiting gas produced by apples and from tubers that had not been exposed to this gas. There is no universal agreement among plant physiologists concerning the significance of catalase activity in plants, but it is generally considered that its intensity is a measure of physiological activity and that it is associated with the function of respiration.

The measurements of catalase intensity were made from sprout and from tuber tissues according to the method adapted by Davis (5). It was found by preliminary tests that the catalase activity of sprouting potato tubers is greatest near the eyes, and tissue surrounding the eyes was consequently used for the tests. To weighed samples of macerated tissue was added 5 cc of hydrogen peroxide, and the amount of oxygen evolved in 5 minutes' time was measured by the displacement of water in a burette. The results obtained and the computed amount of oxygen evolved per 100 mg of tissue are recorded in table 2.

TABLE 2.—*Effect of the growth-inhibiting gas from apples upon catalase activity in the sprout tissue and the tuber tissue of the potato*

Tissue and date	Normal tissue <sup>1</sup>			Affected tissue <sup>2</sup>		
	Weight of sample	Oxygen liberated	Oxygen liberated per 100 mg	Weight of sample	Oxygen liberated	Oxygen liberated per 100 mg
Sprout <sup>3</sup>	Mg	Cc	Cc	Mg	Cc	Cc
Nov. 9, 1932.....	200	29.0	14.50	180	39.0	21.67
Nov. 11, 1932.....	200	34.0	17.00	170	40.2	23.65
Nov. 11, 1932.....	230	36.1	15.69	200	38.8	19.40
Nov. 11, 1932.....	350	37.1	10.60	200	52.0	26.00
Jan. 6, 1934.....	484	23.5	4.86	407	37.7	9.27
Jan. 6, 1934.....	421	14.6	3.47	473	38.7	8.18
Jan. 6, 1934.....	399	35.5	8.90	422	53.8	12.75
Jan. 15, 1934.....	414	41.0	9.90	262	55.1	21.03
Jan. 15, 1934.....	679	56.4	8.36	539	63.1	11.70
Feb. 26, 1934.....	442	39.0	8.82	535	76.4	14.28
Feb. 26, 1934.....	812	76.8	9.46	522	94.5	18.10
Feb. 27, 1934.....	375	53.1	14.16	160	48.5	30.28
Feb. 27, 1934.....	427	72.8	17.05	205	53.3	26.00
Tuber <sup>4</sup>						
Nov. 11, 1932.....	380	41.8	11.00	400	58.6	14.65
Nov. 11, 1932.....	300	32.3	10.77	330	60.1	18.21
Nov. 11, 1932.....	729	50.6	6.94	562	60.2	10.71
Feb. 26, 1934.....	686	72.0	10.50	580	90.1	15.53
Feb. 26, 1934.....	296	33.8	11.42	187	63.8	34.12
Feb. 27, 1934.....	345	52.7	15.28	249	62.3	25.02

<sup>1</sup> Tissues not affected by the growth-inhibiting gas.

<sup>2</sup> Tissues affected by the growth-inhibiting gas.

<sup>3</sup> Percentage increase of oxygen liberated by abnormal over normal, 79.88.

<sup>4</sup> Percentage increase of oxygen liberated by abnormal over normal, 65.27.

The tests on comparative catalase activity indicate that potato sprout and tuber tissues that have been affected by the growth-inhibiting gas from apples contain decidedly more of that enzyme than do the normal tissues. The catalase from abnormal sprout tissue liberated 79.88 percent more oxygen than did this enzyme from the normal sprouts, and the catalase from abnormal tuber tissue liberated 65.27 percent more oxygen than did that from the normal tubers. It is of interest to note that potatoes that had been affected with the growth-inhibiting gas from apples had both a higher respiration intensity and a greater catalase activity than did the normal potatoes.

## OXIDASE ACTIVITY

Comparisons were made of the oxidase activity of potato sprouts that had developed in the presence and in the absence of the growth-inhibiting gas from apples. The measurements of oxidase activity were made according to the simplified method of Bunzel (3).

A higher oxidase activity was recorded from abnormal sprouts than from normal sprouts, as is indicated in table 3. In a test made in January 1934 the oxidase activity from the abnormal sprouts was 11 percent higher than that from the control sprouts, and in a test made 2 weeks later, the oxidase activity from affected tubers was 36 percent higher than that from the control tubers.

TABLE 3.—*Effect of the growth-inhibiting gas from apples upon oxidase activity in the sprout tissue and the tuber tissue of the potato*

Tissue	Oxygen absorbed by—	
	Normal tissue	Abnormal tissue
Sprout.....	Cc 4 899	Cc 5 467
Tuber.....	4 437	6.035

## CHEMICAL ANALYSES OF NORMAL AND AFFECTED SPROUT AND TUBER TISSUES

Chemical analyses were made of normal potato tubers and sprouts, and these analyses were compared with those of similar tissues from potatoes that had been held under the influence of the growth-inhibiting gas from apples. Irish Cobbler tubers were cut into longitudinal halves, and half of each tuber was placed in an enclosed chamber that contained Winesap apples. The tuber halves were covered with a layer of moist sphagnum to provide suitable conditions for growth. The other tuber halves were placed in the control chamber and were covered and held under similar environmental conditions except that no apples were present. The potatoes were allowed to grow approximately 1 month. The sprouts were then removed and dried at 65° to 70° C. after first being heated to between 90° and 100° C. for an hour to stop enzymatic action. Tubers from both lots were sliced and similarly heated and dried.

The analyses recorded in table 4 indicate the variations in composition that existed between the affected and normal sprout and tuber tissues. The total nitrogen content of the compared samples was quite similar. The dried normal and abnormal sprout tissues contained, respectively, 3.70 and 3.66 percent total nitrogen, while the total nitrogen content of the normal and abnormal tuber tissues was respectively 2.12 and 2.11 percent.

TABLE 4.—*Chemical analyses<sup>1</sup> of sprout and tuber tissues from normal potatoes and from potatoes affected by the growth-inhibiting gas from apples*

Tissue	Total nitrogen	Albuminoid nitrogen	Total sugars	Reducing sugars	Starch	Hemicellulose	Crude fiber	Ash	Water
	Percent	Percent	Percent	Percent	Percent	Percent	Percent	Percent	Percent
Sprout.....									
Normal.....	3.70	1.86	15.40	13.46	16.79	5.38	11.65	8.60	91.24
Abnormal.....	3.66	2.61	20.66	17.41	16.67	6.92	7.06	7.00	89.09
Tuber.....									
Normal.....	2.12	1.08	64	0					79.75
Abnormal.....	2.11	1.03	3.20	0					78.11

<sup>1</sup> Water analyses on a fresh-weight basis, other analyses on a dry-weight basis

The albuminoid-nitrogen content of the dried normal and abnormal tuber tissues was respectively 1.08 and 1.03 percent. A greater difference in albuminoid-nitrogen content existed between the affected and the normal sprouts. The normal sprouts contained 1.86 percent of albuminoid-nitrogen, which is 50.27 percent of the total nitrogen content, while the abnormal sprouts contained 2.61 percent of albuminoid nitrogen or 71.31 percent of the total nitrogen content. While the total nitrogen content of the abnormal sprouts was similar to that of the normal sprouts, these analyses show that it was largely in the form of the insoluble albuminoid nitrogen. The abnormal sprouts contained only 57 percent as much protein-free nitrogen as did the normal sprouts.

Total sugars and reducing sugars were more abundant in the abnormal than in the normal sprouts. The normal sprouts contained approximately one-fourth less both of total sugars and of reducing sugars than did the abnormal sprouts.

Tubers that had been exposed to the growth-inhibiting gas for a month contained five times as much total sugars as the untreated control tubers. This accounts for the relatively sweeter taste of the potatoes that were stored with apples. Neither the normal nor the treated potatoes contained reducing sugars.

The dry-weight starch content of the stunted and the normal sprouts was similar, and the hemicellulose content of these sprouts was also similar. No determinations were made of starch, hemicellulose, crude fiber, or ash in the tubers.

The crude-fiber content of the dried normal and abnormal sprouts was, respectively, 11.65 and 7.06 percent, or a decrease of 40 percent in crude fiber in the affected sprout tissue. Ash constituted 8.6 percent of the dry weight of the normal sprouts and 7.0 percent of the dry weight of the abnormal sprouts.

Water constituted 91.24 percent of the normal sprouts and 89.09 percent of the abnormal sprouts before they were dried. The moisture contained in the fresh normal and the treated tubers was, respectively, 79.75 and 78.11 percent.

Abnormal physiological activities, including enzymatic action and synthesis of carbohydrates and proteins, are induced by the growth-inhibiting gas and are apparently related to the morphological changes that occur in affected potato plants. The nature of this relationship has not been demonstrated, but it appears that the unbalance of carbohydrates and proteins within such plants is sufficiently great to affect their morphological development. Botjes (1) observed the premature formation of tubers from the eyes of potatoes from which repeated crops of sprouts had been removed and offered the hypothesis that this abnormal tuber formation is caused by an unbalanced carbohydrate and protein ratio in the mother tuber. He suggested that after repeated crops of sprouts have been removed, a shortage of proteins may finally result, under which conditions the formation of normal sprout tissue is hindered. A hypothesis may likewise be entertained that the gas from apple fruits may cause the permeability of the protoplasm of the potato to be changed, or that because of the effect of this gas on enzymatic action or on other functions, the potato may be unable to supply the developing sprout with the required proportion of carbohydrates and proteins. Analyses of normal and abnormal sprouts indicate that an unbalanced ratio of sugars

and nitrogen compounds occurred in the abnormal sprouts. No great differences occurred in the amount of total nitrogenous materials in the compared sprouts, but albuminoid nitrogen was more abundant in the abnormal sprouts. The abnormal sprouts contained only about 57 percent as much protein-free nitrogen as did the normal ones. Accompanying the reduction in protein-free nitrogen, the abnormal sprouts contained an excess of 34 percent in total sugars and 29 percent in reducing sugars. The effect of this abnormal ratio of carbohydrates and protein-free nitrogen may very probably be the cause of a decided hindrance in normal sprout development.

#### INVESTIGATIONS TO IDENTIFY THE GROWTH-INHIBITING GAS FROM APPLES

Attempts were made in these investigations to identify the volatile growth-inhibiting substance that is produced by apples. The characteristics of this gas were studied from its reaction to certain solvents, absorbents, and oxidizers. Sprouting potato tubers were also treated with the gases of various chemical substances, and the effect of these chemicals upon sprout growth was noted.

A stream of air containing the volatile products from apples was treated with a solvent, absorbent, or oxidizing agent, and then drawn through a closed chamber that contained potatoes planted in moist soil. The index used to determine whether the growth-inhibiting gas had been destroyed was the ability of the potatoes to develop normal sprouts. The duration of the tests was approximately 14 days, which allowed time for sprout growth of the tubers.

The growth-inhibiting gas from apples was not removed or destroyed following dispersion through ethyl alcohol, mineral oil, petroleum ether, toluene, benzene, amylacetate, or potassium hydroxide. Abnormal sprouting of the potatoes occurred after the growth-inhibiting gas had been treated with these substances.

Air containing the gases from apples was passed through an electric furnace by way of a pyrex tube filled with a mixture of 1 part of plat-inized asbestos and 3 parts of black copper oxide. The treated air was then drawn through chambers that contained growing potatoes. The furnace was kept at a glowing-red temperature during the 14-day period of the test. Normal sprout growth resulted from the test potatoes, as is shown in figure 6, *B*, indicating that the growth-inhibiting gas was destroyed under the oxidizing conditions at this temperature.

Activated charcoal (Norit), which had been held at 100° C. for 36 hours before the test, was used in an attempt to adsorb the growth-inhibiting gas by slowly drawing the gases from apples through a 29-inch column of this adsorbent. No evidence was obtained that the growth-inhibiting gas was adsorbed, inasmuch as characteristically inhibited sprouts developed from the indicator potatoes.

Attempts were made to oxidize the growth-inhibiting gas from apples by dispersing it through a 2-percent solution of potassium permanganate made alkaline by the addition of 2 percent of potassium hydroxide. The resultant sprout growth of the tubers was not normal, but slowly elongating, somewhat thickened sprouts that were intermediate between normal and abnormal were produced in one of the tests. Evidently the growth-inhibiting gas was not completely oxidized.

Absorption of the growth-inhibiting gas from apples was attempted with sulphuric acid, fuming sulphuric acid, and chlorosulphonic acid. The gases from apples were passed through these acids in a Bowen potash-absorption bulb. Moisture was removed from the air stream with calcium chloride before the gases entered the absorption bulb, and the acid fumes were removed with a 40-percent potassium hydroxide solution before the air stream entered the potato-growing chambers. The growth-inhibiting gas was destroyed both by fuming

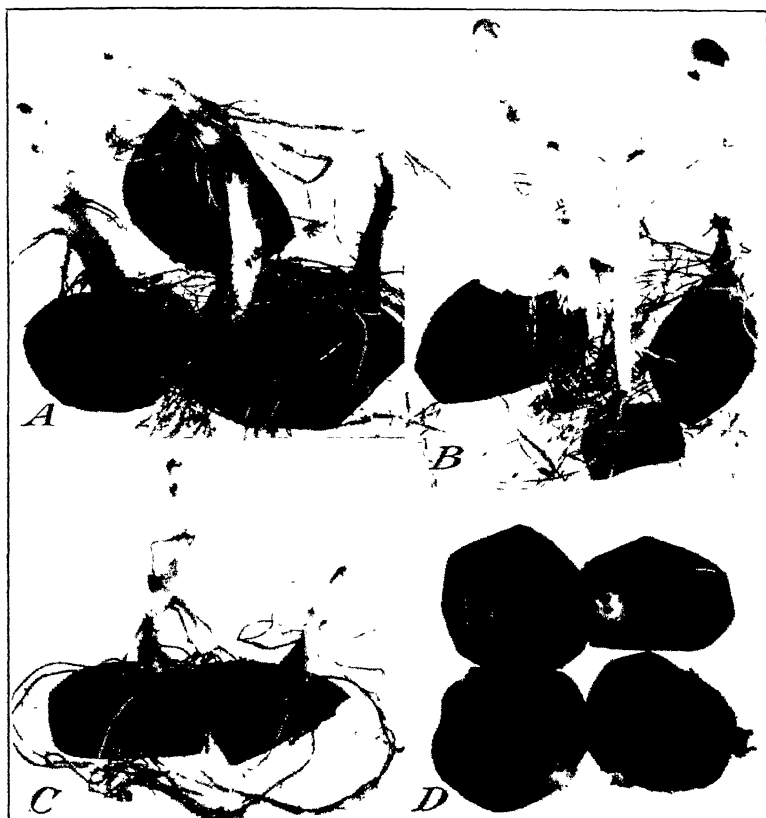


FIGURE 6.—The effect of absorption or oxidation on the growth-inhibiting gas from apples: *A*, Normal sprouts produced after the growth-inhibiting gas had been absorbed with chlorosulphonic acid; *B*, normal sprouts produced after the growth-inhibiting substance had been oxidized under high temperatures; *C*, control, normal sprouts from tubers not subjected to the growth-inhibiting substance; *D*, control, abnormal sprouts from tubers that were supplied with air containing the gases from apple fruits, the air stream being a portion of the supply used for *A* and *B*.

sulphuric acid and by chlorosulphonic acid,<sup>4</sup> as was indicated by the development of normal sprouts from the potatoes that served as indicators (fig. 6, *A*). Sulphuric acid (nonfuming) did not completely absorb the growth-inhibiting gas, and abnormal sprouts developed from the indicator potatoes.

An attempt was made to absorb the growth-inhibiting gas from apples with bromine. The gases from apples were dispersed through

<sup>4</sup>Reported as an absorbent by Botjes (?).

bromine-saturated water, then through a bromine-saturated atmosphere, after which the bromine vapors were stripped from the air stream by dispersion through potassium hydroxide. This absorbent destroyed the growth-inhibiting gas and allowed normal sprouts to develop from the tubers that served as indicators.

In the studies above described, the growth-inhibiting gas from apples was destroyed by substances that are known to react with ethylene. It was oxidized at a high temperature and apparently also by potassium permanganate. Treatments with fuming sulphuric acid, chlorosulphonic acid, and bromine (substances known to be efficient absorbents of ethylene) destroyed its growth-inhibiting property.

Certain effects on the potato plant of the gas from apples are similar to previously described effects of ethylene on other plants, and the possibility that ethylene is the casual growth-inhibiting substance from apples was entertained early in this investigation. Denny (6) found that ethylene caused green lemons to respire from 100 to 250 percent more carbon dioxide than normal, and Regeimbal et al. (9) noted that ethylene hastened the ripening of bananas, in which the liberation of carbon dioxide was doubled or trebled. It was found in the present investigations that approximately 100 percent more carbon dioxide than normal was respired by sprouting potatoes when they were exposed to the growth-inhibiting gas from apples.

Crocker (4) found that ethylene, acetylene, butylene, propylene, and carbon monoxide produce epinasty of the leaves of certain plants. In the present investigation this reaction was produced in potato plants both with ethylene and with the gas from apples.

Above-ground portions of the potato plant were confined in an atmosphere that contained 1 part of ethylene to 20,000 parts of air. The response of the leaves to this gas was similar to the response that followed when potato plants were held in closed containers with apple fruits.

Potato seed pieces planted in moistened sphagnum were confined in a similar concentration of ethylene in order to note the effect of this gas upon the development of sprouts and in order to compare their response with the effect that is produced by the growth-inhibiting gas from apples. The ethylene was renewed at 12-hour intervals after the air in the bell jars that enclosed the experimental potatoes had been completely changed. The effect of ethylene on the potato sprouts was not distinguishable from the effect that is produced by the growth-inhibiting gas from apples. The abnormal sprout growth that developed as a result of the treatment with ethylene is illustrated in figure 7.

Studies were made to identify the growth-inhibiting substance from apples. Fuming sulphuric acid, as was noted above, proved an efficient absorbent of this gas, and it was consequently employed in this investigation. The gases from apples were dispersed continuously for a period of approximately 3 weeks through fuming sulphuric acid in Bowen potash bulbs. The spent acid was made alkaline with sodium hydroxide, refluxed to hydrolyze any ester present, distilled, and the distillate, after being oxidized, tested with the aldehyde fuchsin reagent. A positive aldehyde test was obtained, which suggests that the acid reacted with an olefin, that the resulting ester was hydrolyzed to a primary alcohol, and that the primary alcohol



was oxidized to an aldehyde. This behavior indicates the probability that the growth-inhibiting gas is ethylene<sup>5</sup> and not acetylene, butylene, or propylene. The possibility that the growth-inhibiting gas from apples is acetylene is unlikely because acetaldehyde, formed when the acetylene-sulphuric acid addition product is hydrolyzed, is probably destroyed during the 3-hour period of refluxing in the presence of an alkali. The assumption is made that the growth-inhibiting gas is ethylene rather than any other olefin because the aldehyde

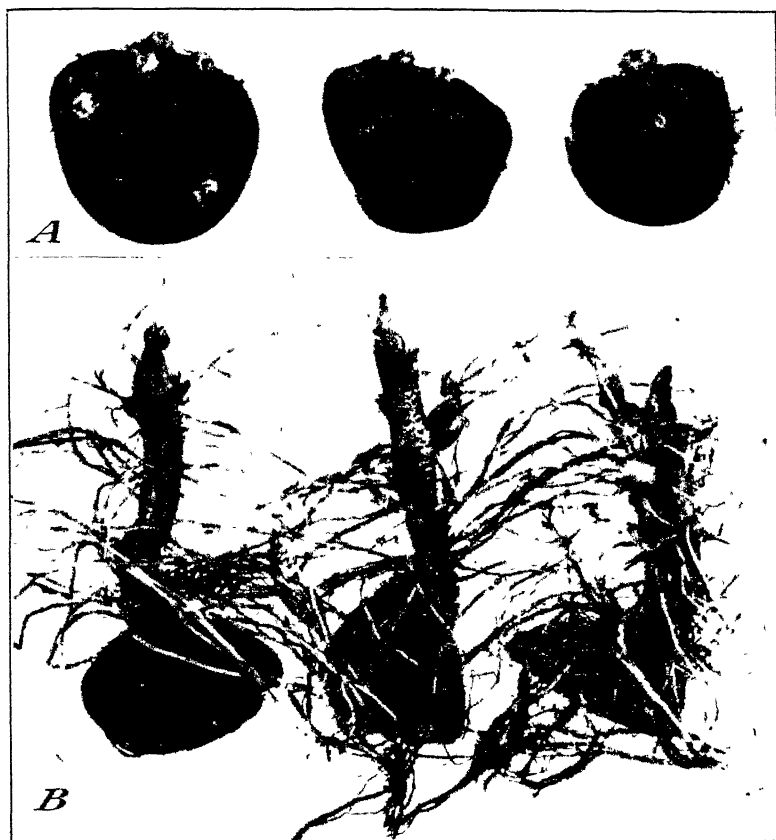


FIGURE 7—Effect of ethylene on potato-sprout production. *A*, Sprout growth from tubers held with ethylene, 1 part to 20,000 parts of air; *B*, control tubers held under a similar environment except that no ethylene was present.

fuchsin test indicated the presence of a primary alcohol, while the homologues of ethylene tend to form secondary and tertiary alcohols after addition reactions and subsequent hydrolysis of resulting esters. Upon oxidation, secondary alcohols yield ketones, which react relatively slowly with the fuchsin reagent, while tertiary alcohols are broken up into ketones and acids.

<sup>5</sup> Gane (8) identified the growth-inhibiting gas from apples as ethylene by employing bromine as the absorbent. The investigations herein described were completed before Gane's report was noted, and they corroborate his conclusions.

## SUMMARY

A volatile substance, normally produced by apple fruits, causes striking morphological and physiological abnormalities in the potato.

This volatile substance is produced by the mesocarp and endocarp of sound ripe apples. Pears and hawthorn fruits also emanate this gas. Affected potato sprouts produce an abnormal radial growth, while longitudinal growth practically ceases, and a tuberlike structure results. Epinasty occurs in affected leaves. Apical growth of the green plant ceases, but radial enlargement results in the younger portions of the stems and in the leaf petioles. Respiration and catalase activity are approximately doubled, the oxidase activity is increased, and a change occurs in the nitrogen-carbohydrate balance of sprouts and tubers.

The sprouting of potatoes in storage can be practically stopped and the tubers can be kept in a well-preserved condition by supplying a sufficient concentration of the growth-inhibiting gas. The total sugar content of the tubers is, however, increased, giving them a sweet flavor. Potatoes to be planted are apparently not injured by the growth-inhibiting gas.

The growth-inhibiting gas is oxidized at high temperatures and with potassium permanganate. It is absorbed by bromine, fuming sulphuric acid, and chlorosulphonic acid.

Analysis of the absorption products obtained by treating the gases from apples with fuming sulphuric acid indicates that ethylene is produced by these fruits.

The effect of ethylene upon sprout development and on the green portions of the potato plant was indistinguishable from the effect that is produced by the growth-inhibiting gas from apples.

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# SEED GERMINATION IN LOBELIA, WITH SPECIAL REFERENCE TO THE INFLUENCE OF LIGHT ON LOBELIA INFLATA<sup>1</sup>

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## INTRODUCTION

The Indian tobacco, *Lobelia inflata* L., a common native annual of eastern North America, is the source of alkaloids used in certain drugs. Most of these alkaloids are obtained from wild plants. Experimental plantings have been made, however, to determine the effect of various treatments on the alkaloidal content of *L. inflata* under cultivation,<sup>3</sup> and in recent years attempts have been made to grow it commercially.

Six years ago several samples of *Lobelia inflata* seed were submitted to the writer with the complaint that when planted the seed had failed to grow. Germination tests on these seeds had also been unsuccessful. The question then arose, Are these seeds immature, dormant, or dead? After subjecting lots of these seeds to various treatments employed to induce germination, such as alternating temperatures, chilling, freezing, soaking in sulphuric acid, hydrochloric acid, or hot water, and removal of seed coats, it was found that some of the samples were dead, and that strong light was necessary to obtain any germination from the others. The exact age of the seed samples was unknown, and no information was available concerning the condition of the seeds at the time of harvest. In order to obtain information on the effect of age upon the conditions necessary for the germination of *L. inflata* an experiment was begun in 1930 with fresh seeds of known age.

## MATERIALS AND PROCEDURE

Seeds of *Lobelia inflata* were obtained from six localities: (1) Marion, Va.; (2) Asheville, N. C.; (3) Statesville, N. C.; (4) Chateaugay, N. Y.; (5) Newport, Vt.; and (6) Lenox, Mass. The first three lots were commercial seeds from the crop of 1929, supplied by J. T. Lloyd of Cincinnati, Ohio. The last three were harvested from wild plants when the fruits were ripe, about the middle of September 1930. These seeds were allowed to air-dry in the laboratory and were stored in a dark case in tightly stoppered glass bottles until they were used for making germination tests.

All tests were made in duplicate with lots of 100 seeds. Preliminary germination tests were made with soil, water, paper towels, and unburned clay flower pots as substrata. The best germination was obtained in water or by placing the seeds on the moistened surface of a clay flowerpot. Small 1-inch pots were used for the germination

<sup>1</sup> Received for publication Jan. 11, 1936; issued May 1936

<sup>2</sup> The writer is indebted to S. R. Patrick and Helen Hazard for assistance in making the germination tests in 1934 and 1935

<sup>3</sup> MASCRÉ, M., and GÉNOT, H. EXPÉRIENCES CULTURALES SUR LA LOBELIE, "LOBELIA INFLATA" L. Bull. Sci. Pharm. 39: 165-172. 1932

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tests. The clay pots were inverted in a glass bowl of water so that the water reached within 5 mm of the surface holding the seeds. The bowls were covered with Petri dishes so as to provide a moist, well-lighted germination chamber. One series of chambers was set in a well-lighted room near a south window; the other series was placed in a dark chamber in the same laboratory room.

The germinators were examined daily and the germinated seedlings were counted and removed on the ninth to twelfth day and at approximately 10-day intervals thereafter for 40 to 60 days. In the earlier tests the germinators in the dark were opened only in a dark room with artificial light, but later, when it was found that the short exposure to daylight did not affect the germination of the soaked seeds, the seeds were examined from time to time in daylight. Germination tests were made of six samples over a period of from 4 to 6 years.

Germination tests of several other species of *Lobelia* were made, by the methods already described, in order to compare the results with those obtained with *L. inflata*. The following lots of seed were harvested in the autumn of 1930 from mature fruits from plants growing wild in various parts of New York: *L. cardinalis* L., *L. dortmanna* L., *L. kalmii* L., and *L. siphilitica* L. In addition, seed of *L. tenuior* R. Br. and five varieties of *L. erinus* L. were obtained from a commercial seed dealer.

#### DATA AND DISCUSSION

The results of germination tests with *Lobelia inflata* seeds from 1 to 6 years of age are recorded in table 1.

TABLE 1.—The germination of *Lobelia inflata* seeds in light and darkness

[100 seeds were used in each test]

Lot no., source of sample, and year of harvest	Year of test	Duration of germination test		Germination in light		Lot no., source of sample, and year of harvest	Year of test	Duration of germination test		Germination in light	
		Begun	Ended					Begun	Ended		
				Per-cent						Per-cent	
1, Marion, Va., 1929	1930	Apr. 17	July 15	16		3, Statesville, N. C., 1929—Continued.	1930	May 12	July 15	80	
	1930	May 1	.....	59			1931	Apr. 12	Apr. 27	81	
	1930	May 12	.....	55			1933	Mar. 16	Apr. 20	44	
	1931	Apr. 12	Apr. 27	67			1934	Mar. 1	Mar. 31	36	
	1933	Mar. 16	Apr. 20	40			1935	Feb. 10	Apr. 8	40	
	1934	Mar. 1	Mar. 31	48		4, Chateaugay, N. Y., 1930	1931	Jan. 2	Feb. 18	0	
	1935	Feb. 10	Apr. 8	28			1933	Mar. 16	Apr. 20	28	
	1930	Apr. 17	July 15	48			1934	Mar. 1	Mar. 31	4	
	1930	May 1	.....	46			1935	Feb. 10	Apr. 8	20	
	1930	May 12	.....	29		5, Newport, Vt., 1930	1931	Jan. 2	Feb. 18	0	
2, Asheville, N. C., 1929	1931	Apr. 12	Apr. 27	55			1933	Mar. 16	Apr. 20	33	
	1933	Mar. 16	Apr. 20	42			1934	Apr. 4	May 4	93	
	1934	Mar. 1	Mar. 31	41			1935	Mar. 29	Apr. 28	94	
	1935	Feb. 10	Apr. 8	34		6, Lenox, Mass., 1930	1931	Jan. 2	Feb. 18	0	
	1930	Apr. 17	July 15	61			1933	Mar. 16	Apr. 20	3	
3, Statesville, N. C., 1929.	1930	May 1	.....	88			1934	Apr. 4	May 4	24	

There was no germination in darkness but when the seeds were exposed to light at the end of the 1935 germination test in darkness the following percentages of germination were obtained for the lot numbers indicated: 1, 36; 2, 23; 3, 68; 5, 97.

All of the samples gave some germination in sunlight. No seeds germinated before the eighth day. Germination usually began on the ninth day, and approximately 80 percent of the total germination occurred between the ninth and the twentieth days. There was

considerable variation in the percentage of germination in the samples of seed from different sources. All six samples continued to remain viable for from 4 to 6 years, or the duration of the experiment. The figures for samples 4, 5, and 6 indicate a period of low germination during the first year, possibly a period of dormancy due to other factors than light. In general, the percentage of germination increased during the second to fourth year and then decreased somewhat.

At no time did even a single seed germinate in the germinators kept in darkness. Some of the lots of seed after having been kept in the dark for 30 to 55 days without germinating were transferred to a room where they were exposed to sunlight, and within 9 to 30 days from 23 to 94 percent germination was obtained (table 1). These germination percentages were very similar to those obtained by placing similar samples of seed directly in the light. These results demonstrate the necessity of light for the germination of seeds of *Lobelia inflata*.

It was thought that if large lots of seeds were placed in germinators, possibly a few seeds would be found to germinate in darkness. Accordingly, lots of several thousand seeds were scattered on the surface of water cultures and placed in dark chambers, and similar control cultures were placed in sunlight. The cultures in the light germinated well, but not a single seed germinated among those kept in the dark.

To determine the influence of light on the germination of *Lobelia inflata* under field conditions, seeds of lots 1, 2, 3, and 5 were sown in rows in a garden (Dunkirk silty clay loam) on May 24, 1934. In one series the seeds were dropped on the surface of the soil and in another they were covered with about 1 cm of finely pulverized soil mixed with sand. No seedlings were visible before June 10, but from then on numerous plants began to appear from the surface-sown seed. Only three plants were found in the rows in which the seed had been covered. It would appear that in growing *L. inflata* from seed good results may be expected by sowing the seed on the surface of a finely prepared seedbed, as is frequently done with tobacco seed.<sup>4</sup> Stockberger,<sup>5</sup> in discussing the planting of *L. inflata* seed states that it is better not to cover them but to sow them on the surface of the soil.

The striking results obtained with *Lobelia inflata* suggested the desirability of testing other species of *Lobelia* to determine whether they would respond to light in the same manner. The results of these tests, recorded in table 2, show that *L. cardinalis* and *L. siphilitica*, like *L. inflata*, failed to germinate in darkness.

The results of the tests with *L. dortmanna* and *L. kalmii* are inconclusive. In *L. dortmanna*, the water lobelia, an aquatic plant of shallow acid ponds and lakes, the seeds failed to germinate, probably because they had been killed by drying. These seeds normally mature in the water, or the capsules, borne on the slightly emerged peduncles, are but little raised above the surface of the water, so that they are kept continually moist until the seeds fall into the water. Seeds produced under such conditions probably do not withstand drying. To test the validity of this explanation, fresh capsules of

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# TIME INTERVAL BETWEEN EGGS OF RHODE ISLAND RED PULLETS<sup>1</sup>

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## INTRODUCTION

The time interval between eggs laid on successive days has been studied by a number of investigators. Warren and Scott<sup>2</sup> have recently reported the results of carefully controlled experiments to obtain more definite information relative to the time element in egg formation. They report for White Leghorns that the average time between eggs laid on successive days is 25.1 hours. Their studies indicate also that the variation between individual birds in time interval between eggs is due largely to variations in the time the egg is held in the uterus.

The studies reported here on Rhode Island Red pullets in the first laying year were undertaken to discover possible relationships between the time between eggs within the clutch of individuals and some important fecundity and reproductive characters.

## DATA RECORDED

Four hundred and nine pedigreed Rhode Island Red pullets bred for high fecundity and hatched from March 8 to May 22, 1926, were trap-nested hourly for a full laying year and the time of laying was recorded on the hour. In other words, eggs taken from the trap-nests from 9 to 10 o'clock were marked as laid at 9 o'clock, and so on throughout the day. Such a method of recording time was reasonably accurate within 1 hour but would in general tend somewhat to increase the recorded time interval over the actual time interval. The mean time interval within clutches from first pullet egg to March 1 was calculated for each individual, together with winter clutch size, winter egg production, hatching season egg weight, hatchability, persistency, and annual egg production. Monthly time between eggs was also calculated for 119 of these birds that were in one house, together with the monthly egg record of each individual.

## CHANGES IN TIME INTERVAL BETWEEN EGGS DURING THE PULLET LAYING YEAR

Figure 1, based on the records of 119 birds, shows clearly that the mean time between eggs laid on successive days varies greatly throughout the first laying year. The great majority of the birds used in this study began their laying year in October. Figure 1 shows that pullets tend to begin their year with a relatively short period between

<sup>1</sup> Received for publication Dec. 9, 1935; issued May 1936. Contribution no. 232 of the Massachusetts Agricultural Experiment Station.

<sup>2</sup> WARREN, D. C., and SCOTT, H. M. THE TIME FACTOR IN EGG FORMATION. *Poultry Sci.* 14: 195-207, illus. 1935

eggs. The mean time interval for October was 26 hours. As the season advances into winter there is a rapid and consistent increase in time between successive eggs through the month of February. The records for March show a pronounced shortening of the time interval, and April is characterized by the shortest time interval of the year, 25.7 hours. As spring advances into summer the time between eggs of a clutch increases and remains at a medium level from June through September. Examination of the individual records shows that the shortest monthly time interval recorded was 23 hours for one bird during June and 23.4 hours for the same individual during April. Several birds showed a monthly mean of 24 hours for April. The maximum time interval recorded was 31.7 hours for one bird during February. The mean time interval for the entire year was 26.49 hours.

The mean monthly egg production of the same group of birds is shown in figure 1. For the winter season up to March 1 there is a

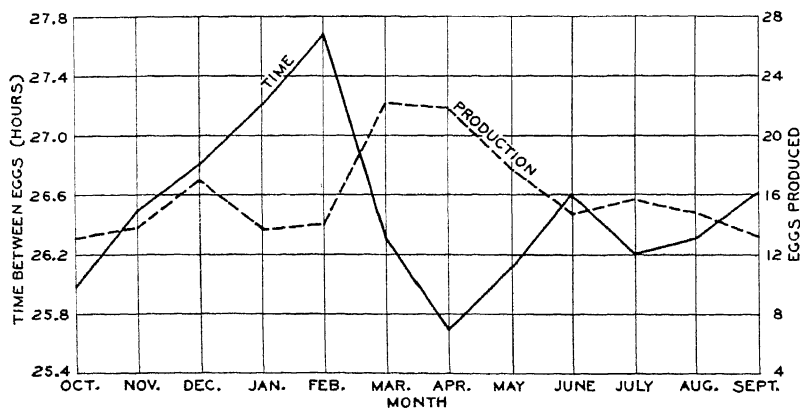


FIGURE 1 —Relation between monthly egg production and monthly interval between eggs, based on the records of 119 birds.

tendency for monthly egg production to decrease as the time between eggs becomes greater. The spring season of normally heavy laying during March, April, and May is accompanied by a shortened interval between eggs. The mean egg production during March was 22.34 and during April it was 21.97. During June, July, and August egg production approaches the mean for the year, which is 16.15 eggs, and the interval between eggs is close to the yearly mean of 26.49 hours.

In general, the data in figure 1 show that heavy egg production is associated with a short interval between successive eggs in a manner similar to the association between large clutch size and high egg production pointed out by Hays and Sanborn.<sup>3</sup>

#### INTERVAL BETWEEN EGGS IN WINTER AS RELATED TO SIZE OF WINTER CLUTCH

Data are available on 409 individuals representing a normal sample for time interval, as figure 2 indicates. The  $\chi^2$  test gave a

<sup>3</sup> HAYS, F. A., and SANBORN, R. TYPES OF INTENSITY IN RHODE ISLAND REDS. Mass. Agr. Expt. Sta. Bull. 286. 11 pp. 1932.



value for *P* of 0.3688. Table 1 presents the mean clutch size of the different time-interval classes for 394 birds. The data show a constant decline in clutch size as the time between eggs increases up to about 28.5 hours. When the time is greater than 28.5 hours, clutch size shows no orderly decrease.

TABLE 1 — *Time between eggs as related to winter clutch size and winter production in 394 birds*

Time between eggs during winter (hours)	Birds			Egg clutch size in winter	Winter egg production	Time between eggs during winter (hours)	Birds			Egg clutch size in winter	Winter egg production	Time between eggs during winter (hours)	Birds			Egg clutch size in winter	Winter egg production
	Number	Number	Number				Number	Number	Number				Number	Number	Number		
24.0...	1	10.50	65.50	26.5...	64	2.77	66.59	29.0...	17	1.41	32.56	31.0...	1	1.00	25.50		
24.5...	17	6.00	85.50	27.0...	53	2.25	60.03	29.5...	5	1.60	35.50						
25.0...	23	5.00	87.24	27.5...	47	2.06	54.86	30.0...	4	1.13	10.50						
25.5...	37	3.64	74.42	28.0...	51	1.85	49.42	30.5...	1	1.50	15.50						
26.0...	50	3.15	76.90	28.5...	23	1.63	41.15										

Figure 3 presents the same data in graphic form. The rate of decrease in clutch size with increased time intervals does not follow a straight line, but rather a hyperbolic curve. The equation of the

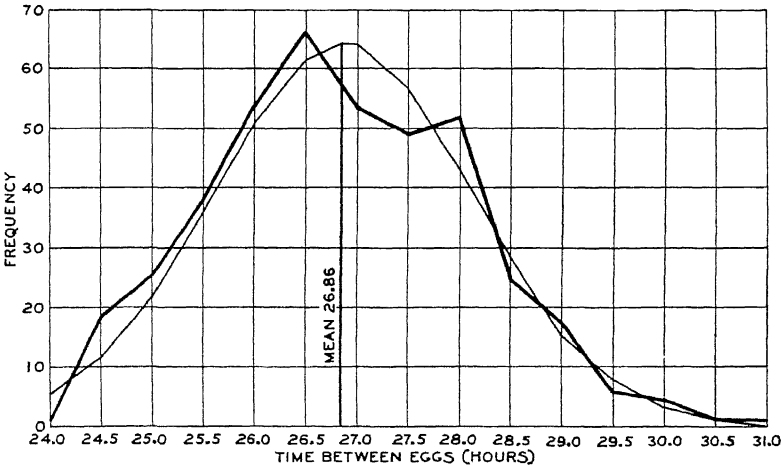


FIGURE 2 — *Frequency distribution of time between eggs during winter (to Mar. 1, 1928) for 409 birds.*

curve shown in the figure is  $y^2 - 0.52xy + 96.67y + 5.82x^2 - 326.19x = -4,656.01$ . This curve fits the actual data very closely for the clutch-size classes that correspond with time-interval classes up to 28.5 hours. This includes 366 out of a total of 394 birds.

These data furnish evidence that large clutch size definitely depends on short time intervals between eggs and that clutch size does actually measure the rate of functioning of the female reproductive system. The mean winter time interval was 26.9 hours.

### INTERVAL BETWEEN EGGS IN WINTER AS RELATED TO WINTER EGG PRODUCTION

In table 1 is shown the mean winter egg production for the different classes of layers with respect to time between eggs. There is a generally consistent and regular decline in winter egg production as the time between eggs increases. With an increase in time from 24.5 hours to 29.5 hours there was a decrease in winter egg production from 85.5 eggs to 35.5 eggs. These data indicate that any increase in

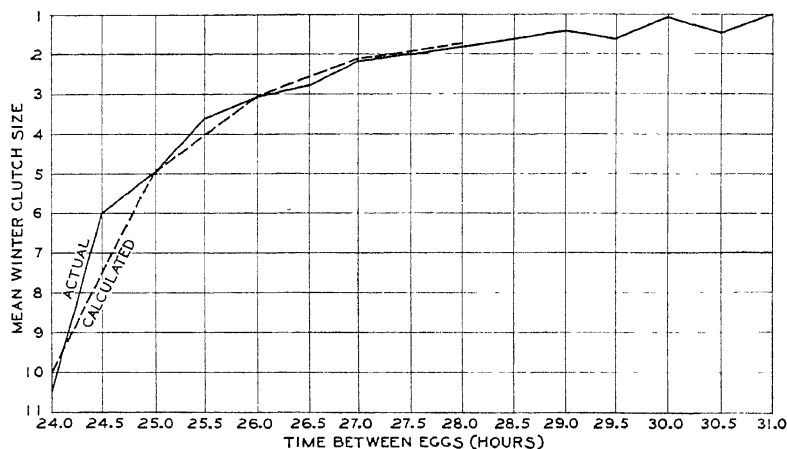


FIGURE 3—Actual and calculated time between eggs during winter, as related to mean size of clutch, for 394 birds

the time interval between eggs in a clutch is likely to be followed by a significant decline in egg production.

### INTERVAL BETWEEN EGGS IN WINTER AS RELATED TO HATCHING-SEASON EGG WEIGHT

A group of 47 of these birds was used for breeding in the spring of 1927. The eggs were weighed during the last week of February and the first week of March to secure an approximation of the egg weight of each bird during the hatching season extending from February 22 to March 20.

TABLE 2.—Time between eggs as related to egg weight during hatching season and to hatchability in 47 birds used for breeding

Time between eggs during winter (hours)	Birds	Egg weight during hatching season	Hatchability of eggs	Time between eggs during winter (hours)	Birds	Egg weight during hatching season	Hatchability of eggs
	<i>Number</i>	<i>Grams</i>	<i>Percent</i>		<i>Number</i>	<i>Grams</i>	<i>Percent</i>
24.5.....	3	50.3	35.0	26.5.....	11	54.0	58.0
25.0.....	8	52.9	63.8	27.0.....	2	52.0	50.0
25.5.....	11	55.2	50.5	27.5.....	2	52.5	35.0
26.0.....	10	54.8	52.3				

In table 2 the mean egg weight of the birds classified with respect to time interval is presented. As the table shows, no birds were used for breeding that had a time interval greater than 27.5 hours; all were, therefore, intense layers. The maximum egg weight of 55.2 g occurred in the group with an average interval of 25.5 hours. When the interval was less than 25.5, or more than 26.5 hours, egg size was significantly below average. These limited data confirm the idea that very rapid laying is likely to be associated with small eggs, as Hays<sup>4</sup> has pointed out.

#### INTERVAL BETWEEN EGGS IN WINTER AS RELATED TO HATCHABILITY, PERSISTENCY, AND ANNUAL PRODUCTION

The relation between the mean time between eggs up to March 1 and percentage of fertile eggs that hatched is set forth in table 2. There is some evidence that the eggs of birds that lay successive eggs at 24.5- or 27.5-hour intervals may be low in hatchability. This is in agreement with the findings of Hays and Nicolaides<sup>5</sup> that birds laying eggs developed only to the pregastrula or early gastrula stage are likely to have low records of hatchability. The data further tend to show that when the eggs are laid at intervals greater than 27 hours the hatchability may be low. The most satisfactory hatchability was obtained from individuals having a mean time interval from 25 to 26.5 hours.

Persistency records covering the 365-day laying year are available for 246 birds. In table 3 the mean persistency record for the different winter season time-interval classes is recorded. The data show a consistent decrease in persistency with each increase in winter time interval up to about 27.5 hours, after which the decrease is less orderly. The data point to an important association between rapid rate of laying in winter and persistent laying at the end of the year. Such a relation is of considerable economic importance.

TABLE 3.—Time between eggs as related to annual persistency and annual egg production in 246 birds

Time between eggs during winter (hours)	Birds	Annual persistency	Annual egg production	Time between eggs during winter (hours)	Birds	Annual persistency	Annual egg production
	Number	Days	Number		Number	Days	Number
24.5.....	13	352 38	238 92	27.5.....	27	330 52	186.07
25.0.....	18	337 00	228.67	28.0.....	29	330 10	205.10
25.5.....	25	335 00	216 00	28.5.....	14	317 36	165.57
26.0.....	32	330 75	215 91	29.0.....	7	287 00	169.14
26.5.....	43	328 28	209 09	29.5.....	3	303 67	162 00
27.0.....	35	325 57	194 14				

The relation between time interval between eggs for the winter season and annual egg production is also shown in table 3. There is a generally regular decline in egg production with each increase in interval between eggs. In other words, the ability to manufacture eggs in rapid succession during the winter season is a very good indi-

<sup>4</sup> HAYS, F. A. INCREASE IN EGG WEIGHT DURING THE PULLET LAYING YEAR. Poultry Sci. Assoc. Proc. (1930) 22, 16-19, illus. 1931

<sup>5</sup> HAYS, F. A., and NICOLAIDES, C. VARIABILITY IN DEVELOPMENT OF FRESH-LAID HEN EGGS. Poultry Sci. 13, 74-80, illus. 1934.

cation that the bird will have a high annual egg record. The data show that an increase in time interval between eggs from 24.5 to 28.5 hours gave a decline in annual egg record from 239 eggs to 166 eggs. As the time interval becomes greater than the population mean of 26.9 hours there is a slight falling off in the rate of decrease in egg production in relation to increase in time interval.

#### SUMMARY

The mean time interval between eggs laid on successive days was calculated on 394 Rhode Island Reds from first pullet egg up to March 1. Monthly time intervals were determined on 119 of these birds which were housed together. A study was made of the relation of the time interval between successive eggs to several fecundity and reproductive characters. A number of significant relationships were observed, as follows:

(1) Monthly time interval between eggs reached the lowest level during April and stood at a relatively low level during the months of maximum egg production. The greatest time interval occurred during months of lowest production.

(2) Short time intervals between eggs for the winter season were characteristic for birds showing large clutch size.

(3) Short time intervals between eggs up to March 1 were characteristic of heavy winter producers.

(4) A mean winter season time interval of 25.5 hours or more was associated with maximum egg weight during the hatching season. When the time interval fell below 25.5 hours or was above 26.5 hours, there was a decrease in egg size.

(5) There was some evidence that a winter time interval as low as 24.5 hours may be associated with low hatchability.

(6) In the birds studied each increase in winter time interval tended to be followed by decreased persistency.

(7) Short winter time interval between eggs was definitely associated with high annual egg production. The winter time interval appears to have definite value in predicting what the egg record may be at the close of the pullet laying year.

# ACCURACY OF THE DETERMINATION OF LEAD AND ARSENIC ON APPLES<sup>1</sup>

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## INTRODUCTION

The accuracy of the Gutzeit method for the determination of small amounts of arsenic has been studied by several workers (2, 3, 4, 8),<sup>2</sup> but the accuracy of the determination of similar small amounts of lead has apparently not been investigated. Federal and State regulatory bodies have now concerned themselves with the amount of lead present on edible fruit as spray residue and have set up limits which legally may not be exceeded.

The legal limits for lead and arsenic trioxide during the season of 1935 have been set at 0.018 and 0.010 grain per pound, respectively. In view of these limits, and the fact that the limits for lead are being gradually reduced, the accuracy of the determinations of these elements becomes important to the chemist, for the analytical results must be interpreted in relation to these legal tolerances.

Any study of the accuracy of these determinations naturally divides itself into two parts: The accuracy of the sampling, and the accuracy of the determination itself. The summation of the errors in these two phases of the work represent the errors in the final determination.

Lead, when present on the surface of apples as spray residue, may combine chemically with the waxy coating of the fruit (5), and thus it presents a different problem from arsenic, which apparently does not so combine. In this study the accuracy of the chemical determination of lead will be discussed first, followed by a discussion of the accuracy of the estimation as a whole (including sampling) of both lead and arsenic. The accuracy of the chemical determination of arsenic by the Gutzeit method has been discussed at length (4, 7), and will not be considered here.

## ACCURACY OF THE CHEMICAL DETERMINATION OF LEAD BY THE PHOTOELECTRIC METHOD

The photoelectric method for the determination of lead on fruit, proposed by Frear and Haley (6), is briefly described in order that its advantages and limitations may be better understood.

Light from an electric bulb, regulated by a suitable rheostat, is allowed to shine upon the surface of a photronic cell (Weston no. 594), which is connected directly to a microammeter having a capacity of 200 microamperes. Between the source of light and the photronic cell is interposed a cylindrical glass tube containing the solution to be analyzed for lead. The reading of the microammeter is brought to the maximum of the instrument through an adjustment of the

<sup>1</sup> Received for publication Nov. 13, 1935, issued May 1936. Technical Paper No. 647 of the Pennsylvania Agricultural Experiment Station

<sup>2</sup> Reference is made by number (italic) to Literature Cited, p. 644.

intensity of the light by means of the rheostat. To the solution in the tube is then added sufficient sodium sulphide to precipitate completely the lead present as colloidal lead sulphide. The brown colloidal precipitate reduces the amount of light passing through the solution and falling upon the surface of the photonic cell. The relation between the amount of light transmitted, as measured by the microammeter connected with the cell, and the amount of lead present may be easily determined by standardization with known amounts of lead.

It is apparent that two sources of error are inherent in the method: The sum of the errors due to variation in technique, reagents, etc., which may be called the error of operation; and the error of standardization.

To determine the probable error of a single or of the mean of duplicate determinations at a level which will fall within the range of practical work, 50 determinations were made on solutions each containing 0.0004 g of lead as lead acetate (table 1). These solutions were each carried through the entire analytical procedure, including the digestion with concentrated sulphuric and nitric acids, which is used on samples of apple wash to destroy the organic material. The quantity of lead was selected at 0.0004 g since this is equivalent to a concentration of 0.020 grain of lead per pound of apples when an aliquot of the apple wash representing 140 g of apples is taken for analysis.

TABLE 1.—Analyses of 50 aliquot portions of solution, each of which contained 0.4000 mg of lead

Sample no	Micro-ammeter reading	Milli-grams of lead	Sample no.	Micro-ammeter reading	Milli-grams of lead	Sample no.	Micro-ammeter reading	Milli-grams of lead
1.....	54	0.400	18.....	56	0.393	35.....	53	0.404
2.....	52	410	19.....	53	.404	36.....	55	.396
3.....	52	410	20.....	54	.400	37.....	54	.400
4.....	54	.400	21.....	52	.410	38.....	54	.400
5.....	55	.396	22.....	56	.393	39.....	51	.400
6.....	56	.393	23.....	57	.390	40.....	55	.396
7.....	53	.404	24.....	53	.404	41.....	54	.400
8.....	53	.404	25.....	54	.401	42.....	53	.404
9.....	53	.404	26.....	54	.400	43.....	55	.396
10.....	54	.400	27.....	52	.410	44.....	54	.400
11.....	55	.396	28.....	56	.393	45.....	56	.393
12.....	55	.396	29.....	56	.393	46.....	53	.404
13.....	54	.400	30.....	53	.404	47.....	52	.410
14.....	55	.396	31.....	52	.410	48.....	55	.396
15.....	56	.393	32.....	55	.396	49.....	54	.400
16.....	55	.396	33.....	54	.400	50.....	53	.404
17.....	56	.393	34.....	55	.396			

By the use of the formula  $P. E. = \frac{0.6745\sigma}{\sqrt{n}}$ , in which the standard

deviation is calculated by the formula  $\Sigma = \sqrt{\frac{D^2}{n}}$ , the probable error of the microammeter readings obtained by analyzing the 50 samples was calculated. The mean value was  $54 \pm 0.13$  microamperes, with a probable error of a single determination of  $\pm 0.89$  microampere, and of the mean of duplicate determinations of  $\pm 0.63$  microampere. Since with the instrument used it was not possible to make readings more closely than 1 microampere, the probable error of even a single determination was less than a measurable value.

To translate the microammeter readings into terms of lead concentration, a standardization curve was used which had been constructed by using the mean of 10 determinations at 5 different concentrations. The microammeter reading on this curve corresponding to 0.0004 g of lead was 54 microamperes. By the same formulae as above, the calculated probable error of a single determination was  $\pm 0.0036$  mg; and that of the mean of duplicate determinations was  $\pm 0.0026$  mg.

The probable error of the determination as a whole is further affected, by definition, by any error in the preparation of the curve of standardization. To include this factor, the formula  $P. E. = \sqrt{(P. E._s)^2 + (P. E._{10})^2}$ , in which  $P. E.$  is the probable error of the method as a whole,  $P. E._s$  is the probable error of a single determination, and  $P. E._{10}$  is the probable error of the 10 determinations made to establish the point of reference on the curve of standardization. When the calculation is carried out, the true probable error of a single determination is  $\pm 0.0038$  mg. When the probable error of duplicate determinations is calculated, the value is  $\pm 0.0028$  mg.

A comparison of the relative accuracy of the lead method with that of the modified Gutzeit method for the determination of arsenic studied by Barnes and Murray (4) indicates that when the quantities determined are considered the method for lead exhibits a relatively higher accuracy than the method for arsenic. Barnes and Murray state that with a solution containing 0.0273 mg of  $As_2O_3$ , the probable error of duplicate determinations was  $\pm 0.0023$  mg and, when the probable error of standardization was considered, the true probable error of the determination was  $\pm 0.0025$  mg, the mean of 10 individuals being used to locate each point on the standardization curve. The corresponding figures found for the lead determination, in which the standard solution contained 0.4000 mg, calculated in the same manner, were respectively  $\pm 0.0026$  mg and  $\pm 0.0028$  mg.

#### ACCURACY OF THE FINAL RESULTS FROM THE ANALYSIS OF LEAD AND ARSENIC

To include the errors due to sampling, which obviously affect the accuracy of the final values for lead and arsenic, the procedure described below was followed.

Samples of apples, ranging in weight from approximately 7 to 20 kg, were selected, usually picked from the lower limbs of trees in the orchards. Ordinary precautions were taken in handling, and apples of nearly the same size were selected. These samples were taken to the laboratory, and from them two lots of 700 g each were taken, and each washed thoroughly with boiling 1.5-percent hydrochloric acid with 1.0 percent of sodium chloride added. The washings, when cool, were made up to 500 ml with acid of the same concentration. The lead was determined on an aliquot by the method described above (6), and arsenic was determined by the Gutzeit method as described in the Official Methods (1). In all, 164 lots were analyzed, with a wide range of lead and arsenic content. The results are given in table 2. For convenience, the first of the duplicate lots is designated as A, the second B.

The mean  $As_2O_3$  content of the A samples was 0.00950 grain per pound, and of the B samples 0.00938 grain per pound. The mean of

the average of the A and B samples was 0.00944 grain, and the average deviation was 0.00070 grain, or 7.4 percent of the mean. For lead, the mean of the A samples was 0.02255 grain per pound, and for the B samples 0.02235 grain, while the mean of the averages of the A and B samples was 0.02246 grain per pound. The average deviation was 0.00152 grain of lead per pound, or 6.8 percent of the mean.

TABLE 2.—Arsenic and lead (grain per pound) <sup>1</sup> found in duplicate samples of apples

Sample	Arsenic as As <sub>2</sub> O <sub>3</sub>				Lead			
	A	B	Mean	Deviation	A	B	Mean	Deviation
B2.....	0.001	0.001	0.0010	0.0000	0.003	0.005	0.0040	0.0010
B17.....	.006	.007	.0065	.0005	.016	.018	.0170	.0010
B20.....	.016	.016	.0160	.0000	.028	.028	.0280	.0000
B21.....	.010	.011	.0105	.0005	.022	.026	.0240	.0020
B23.....	.001	.001	.0010	.0000	.003	.003	.0030	.0000
B25.....	.002	.002	.0020	.0000	.007	.008	.0075	.0005
B26.....	.012	.009	.0105	.0015	.028	.021	.0260	.0020
B27.....	.002	.002	.0020	.0000	.005	.013	.0090	.0040
B29.....	.008	.006	.0070	.0010	.022	.016	.0190	.0030
B30.....	.012	.010	.0110	.0010	.029	.027	.0280	.0010
B33.....	.018	.018	.0180	.0000	.033	.034	.0335	.0005
B34.....	.010	.012	.0110	.0010	.026	.026	.0260	.0000
B40.....	.005	.005	.0050	.0000	.013	.010	.0115	.0015
B46.....	.032	.032	.0320	.0000	.067	.077	.0720	.0050
B47.....	.042	.042	.0420	.0000	.082	.080	.0810	.0010
B48.....	.015	.016	.0155	.0005	.035	.039	.0370	.0020
B49.....	.007	.006	.0065	.0005	.013	.010	.0115	.0015
B51.....	.026	.024	.0250	.0010	.046	.055	.0505	.0045
B52.....	.007	.008	.0075	.0005	.021	.022	.0215	.0005
B59.....	.002	.002	.0020	.0000	.006	.008	.0070	.0010
B139.....	.006	.005	.0055	.0005	.018	.015	.0165	.0015
B146.....	.006	.006	.0060	.0000	.013	.016	.0145	.0015
B148.....	.011	.013	.0120	.0010	.032	.035	.0335	.0015
B149.....	.009	.011	.0100	.0010	.022	.020	.0210	.0010
B150.....	.012	.012	.0120	.0000	.035	.026	.0305	.0045
B152.....	.009	.008	.0085	.0005	.037	.025	.0310	.0060
B155.....	.011	.011	.0110	.0000	.040	.038	.0390	.0010
B224.....	.003	.002	.0025	.0005	.006	.004	.0050	.0010
B228.....	.001	.001	.0010	.0000	.003	.002	.0025	.0005
B230.....	.001	.002	.0015	.0005	.003	.005	.0040	.0010
B231.....	.002	.003	.0025	.0005	.005	.009	.0070	.0020
B232.....	.005	.005	.0050	.0000	.012	.007	.0095	.0025
B233.....	.005	.005	.0050	.0000	.012	.015	.0135	.0015
B234.....	.003	.002	.0025	.0005	.006	.004	.0050	.0010
B238.....	.011	.012	.0115	.0005	.028	.032	.0300	.0020
B239.....	.007	.004	.0055	.0015	.010	.008	.0090	.0010
A36.....	.005	.004	.0045	.0005	.011	.013	.0120	.0010
A37.....	.007	.006	.0065	.0005	.021	.019	.0200	.0010
A38.....	.005	.005	.0050	.0000	.010	.014	.0120	.0020
A39.....	.003	.003	.0030	.0000	.010	.008	.0090	.0010
A40.....	.004	.002	.0030	.0010	.004	.006	.0050	.0010
A41.....	.005	.007	.0060	.0010	.015	.011	.0130	.0020
A42.....	.012	.013	.0125	.0005	.038	.038	.0380	.0000
A43.....	.009	.010	.0095	.0005	.019	.021	.0200	.0010
A44.....	.011	.015	.0130	.0020	.034	.030	.0320	.0020
A45.....	.015	.017	.0160	.0010	.044	.048	.0460	.0020
A46.....	.007	.008	.0075	.0005	.018	.021	.0195	.0015
A47.....	.004	.004	.0040	.0000	.010	.010	.0100	.0000
A48.....	.007	.008	.0075	.0005	.019	.016	.0175	.0015
A49.....	.015	.010	.0125	.0025	.026	.026	.0260	.0000
A50.....	.006	.010	.0080	.0020	.032	.033	.0325	.0005
A57.....	.006	.007	.0065	.0005	.016	.020	.0180	.0020
A58.....	.004	.008	.0060	.0020	.014	.016	.0150	.0010
A59.....	.014	.009	.0115	.0025	.030	.026	.0280	.0020
A60.....	.009	.008	.0085	.0005	.021	.022	.0215	.0005
A61.....	.010	.009	.0095	.0005	.029	.032	.0305	.0015
A62.....	.007	.007	.0070	.0000	.018	.019	.0185	.0005
A63.....	.013	.012	.0125	.0005	.032	.036	.0340	.0020
A65.....	.010	.008	.0090	.0010	.025	.022	.0235	.0015
A66.....	.011	.011	.0110	.0000	.037	.019	.0180	.0010
A67.....	.011	.012	.0115	.0005	.031	.027	.0290	.0020
A81.....	.011	.009	.0100	.0010	.028	.025	.0265	.0015
A223.....	.014	.012	.0130	.0010	.029	.025	.0270	.0020

<sup>1</sup> Duplicate lots A and B.



TABLE 2.—Arsenic and lead (grain per pound) found in duplicate samples of apples—Continued

Sample	Arsenic as As <sub>2</sub> O <sub>3</sub>				Lead			
	A	B	Mean	Deviation	A	B	Mean	Deviation
A224.....	0.012	0.010	0.0110	0.0010	0.020	0.019	0.0195	0.0005
A225.....	.008	.009	.0085	.0005	.019	.020	.0195	.0005
A226.....	.019	.014	.0165	.0025	.025	.015	.0200	.0050
A227.....	.018	.012	.0150	.0030	.025	.021	.0230	.0020
A228.....	.012	.017	.0145	.0025	.020	.022	.0210	.0010
A233.....	.007	.009	.0080	.0010	.022	.017	.0195	.0025
A261.....	.020	.020	.0200	.0000	.032	.029	.0305	.0015
A304.....	.009	.010	.0095	.0005	.022	.026	.0240	.0020
A305.....	.005	.004	.0045	.0005	.014	.014	.0140	.0000
A306.....	.009	.009	.0090	.0000	.016	.014	.0150	.0010
A307.....	.004	.003	.0035	.0005	.012	.012	.0120	.0000
A312.....	.004	.004	.0040	.0000	.012	.014	.0130	.0010
A313.....	.002	.004	.0030	.0010	.008	.010	.0090	.0010
A316.....	.006	.004	.0050	.0010	.012	.010	.0110	.0010
A317.....	.003	.004	.0035	.0005	.011	.010	.0105	.0005
A318.....	.040	.035	.0375	.0025	.100	.090	.0950	.0050
A319.....	.024	.020	.0250	.0010	.075	.078	.0765	.0015
A322.....	.003	.006	.0045	.0015	.008	.012	.0100	.0020
A323.....	.003	.003	.0030	.0000	.009	.007	.0080	.0010
Average.....	.00950	.00938	.00944	.00070	.02255	.02235	.02246	.00152

The ratio Pb/As<sub>2</sub>O<sub>3</sub> in the mean values is 2.37/1, and the ratio average deviation Pb/average deviation As<sub>2</sub>O<sub>3</sub> is 2.15/1. These ratios, as well as the percentage values given in the previous paragraph, indicate that the error of the lead determination is not so great as that of the arsenic determination. Apparently the error of sampling affects the accuracy of the determination of these elements to much the same degree, and the increased accuracy of the lead determination reflects the greater accuracy of the chemical part of the procedure, mentioned previously. It is to be noted that the mean values for both arsenic and lead are close to the amounts permitted by law, and therefore the estimations of accuracy should be particularly applicable to samples falling in this range.

### SUMMARY AND CONCLUSIONS

The errors incident to the determination of lead and arsenic as spray residues on apples are due to two sources: Errors of sampling and errors in the chemical determination of the elements. The latter may be further divided into errors of technique and errors of standardization.

When the chemical determination of lead by the photoelectric method is considered separately, the probable error was  $\pm 0.0028$  mg, when samples each containing 0.4000 mg of lead were analyzed. For the entire procedure, including both the chemical determination and the sampling, the mean amount of As<sub>2</sub>O<sub>3</sub> present was 0.00944 grain per pound of fruit, with an average difference between duplicates of 0.00140 grain per pound. For lead the mean values were 0.02246 grain per pound of fruit, with an average difference between duplicates of 0.00304 grain per pound. These are the mean values of 164 apple samples analyzed in duplicate.

The lead determination was slightly more accurate than the Gutzeit arsenic estimation, even when the errors due to sampling were

considered. The average deviation of individual arsenic determinations from the mean of duplicate determinations was 7.4 percent of the mean; for lead it was 6.8 percent of the mean. These data are based on the analysis of 164 samples

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## THE DIURNAL CYCLE OF THE POWDERY MILDEW *ERYSIPHE POLYGONI*<sup>1</sup>

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### INTRODUCTION

Studies of the strains of *Erysiphe polygoni* DC. parasitizing red clover (*Trifolium pratense* L.), cabbage (*Brassica oleracea capitata* L.), and larkspur (*Delphinium* sp.) have shown that certain phases of fungus activity regularly occur at a certain time during the day. This developmental rhythm is called the diurnal cycle of *E. polygoni*, and its manifestation with respect to the germinability of the conidia, the formation of appressoria, and the formation and dissemination of conidia is described herein. Certain other phenomena, such as the time of day at which infection may occur and the transpiration of diseased leaves, also show a diurnal cycle. The cause and some of the limitations of this cycle are discussed.

### GERMINABILITY OF CONIDIA

Conidia from field clover plants were shaken onto dry glass slides at 2- or 3-hour intervals throughout the day. Of four slides of conidia collected at each time, two were left fully exposed to the natural environment in the field and the other two were placed in Petri dish moist chambers held at 23° C. in the dark. Of 200 or more conidia on each slide, those germinating were counted on one slide of each pair after 3 hours (germination interval) and on the other slide after 6 hours. The results of one such test on August 22 to 23, 1933, are given in columns 2, 3, 4, and 5 of table 1. In this, as in other tests, conidia taken from field plants during the light portion of the day gave a higher germination than those taken at night. Conidia collected during the night, late morning, and early afternoon and exposed in the field showed little or no germination after either 3 or 6 hours; those exposed to the heat of the day were severely shriveled, but those exposed at night, when the temperature was much lower, remained turgid. Conidia collected between 10 a. m. and 8 p. m. germinated well in the moist chambers at 23° C., while those collected at night germinated poorly or not at all. There was but little increase in germination after 3 hours.

In several tests the cycle of germinability of conidia from field plants was compared in light and in darkness. The spore mounts exposed to light were in Petri dish moist chambers 93 cm from eight

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300-watt lamps, in a constant environment of 22° C. The mounts in darkness were in light-proof paper boxes beside the exposed mounts. The temperature in the boxes was 1° to 2° higher than the outside temperature, but this slight difference probably had little or no effect on the results. The results of one of these tests are given in columns 6 and 7 of table 1. The conidia taken in the high phase of the germination cycle (noon to 7 p. m.) germinated almost as well in darkness as in light, but conidia taken in the low phase of the cycle were greatly stimulated by light.

TABLE 1.—Effect of time of collection, of germination interval, and of environment on the germination of conidia of *Erysiphe polygoni* from field clover plants, Madison, Wis., 1933

Time of making spore mount	Germination of conidia after indicated treatment on—					
	Aug. 22-23				Aug. 29-30	
	Spore mounts exposed in the field for—		Spore mounts held at 23° C. in dark moist chambers for—		In artificial light for 3 hours at 22° C.	In darkness for 3 hours at 23°-24° C.
	3 hours	6 hours	3 hours	6 hours		
	Percent	Percent	Percent	Percent	Percent	Percent
1 p. m.-----	10	10	50	57	79	40
3 p. m.-----	10	10	52	87	64	48
5 p. m.-----	51	83	56	64	38	25
7 p. m.-----	4	6	79	53	-----	-----
8 p. m.-----	1	3	24	26	-----	-----
9 p. m.-----	-----	-----	-----	-----	76	6
10 p. m.-----	0	0	6	6	-----	-----
12 p. m.-----	-----	-----	-----	-----	32	0
1 a. m.-----	0	0	1	4	-----	-----
3 a. m.-----	-----	-----	-----	-----	18	1
4 a. m.-----	0	0	0	1	-----	-----
6 a. m.-----	-----	-----	-----	-----	29	3
7 a. m.-----	0	10	0	9	-----	-----
9 a. m.-----	-----	-----	-----	-----	29	1
10 a. m.-----	10	10	15	70	-----	-----
12 m.-----	-----	-----	-----	-----	89	32
1 p. m.-----	10	10	43	64	-----	-----
3 p. m.-----	-----	-----	-----	-----	83	72

<sup>1</sup> Conidia severely shriveled.

The cycle of germinability of conidia from plants growing in the greenhouse and of conidia from plants growing in environmental control chambers was similar to that already described for conidia from plants growing in the field. In the greenhouse during these tests the temperature was nearly constant at 20° C., the relative humidity was not measured, and the duration of light was similar to that prevailing outdoors. In the control chambers the plants were grown at 20°, 60 percent relative humidity, and in a 12-hour day (12 hours' continuous light alternated with 12 hours' continuous darkness) with about 1,050 foot-candles of light at the level of the plants.

Age of conidia was not found to influence the cycle of germinability. In one test conidia from mildewed greenhouse plants from which most of the mature conidia had been removed by shaking the plants 24 hours before starting the germination test showed the same cycle of germinability as conidia from plants on which the conidia had been allowed to accumulate for 4 days.

Several attempts were made to increase the germination of conidia taken in the low phase of the germinability cycle. Exposure to light greatly increased the germination of these conidia, as already men-

tioned. Placing the conidia on sugar solution, placing the petioles of the mildewed leaves in 1-percent sucrose solution for 4 hours before making the germination test, and the presence of healthy host leaves in the germination chamber had no effect on the germination of conidia, but in each of five tests the germination of conidia was greatly increased by placing the conidia on host leaves. Excised leaflets from healthy greenhouse plants and dry slides were placed at random in the bottom of a large can, and conidia from mildewed greenhouse plants were dusted on them from above. The average number of conidia counted per unit area on the slide was assumed to be the same on the leaflets. The leaflets and slides were placed in Petri dish moist chambers during the germination interval. The germinated and the ungerminated conidia on the glass slides were counted after 9 hours. After the same period, which was sufficient to allow leaf penetration by the haustoria from the germinated conidia, the leaflets were fixed in a solution of alcohol and acetic acid, stained with acid fuchsin, and examined. The number of established young colonies per unit area of leaves was divided by the original number of conidia per unit area, as determined from the glass slides, to determine the percentage of germination on the leaves. The value obtained in this way was low because many germinated conidia were washed away in the staining process, but the results (table 2) show that in each case the amount of germination on host leaves was much greater than that on glass slides.

TABLE 2.—*Effect of host leaves on germination of conidia, Madison, Wis., 1933*

Germination interval	Germination of conidia on—	
	Dry glass slides in dark moist chambers	Excised clover leaflets in dark moist chambers
	Percent	Percent
12 p. m., Nov. 6, to 9 a. m., Nov. 7.....	4 2	19.20
12 p. m., Nov. 12, to 11 a. m., Nov. 13.....	.0	.11
11 p. m., Nov. 23, to 9 a. m., Nov. 24.....	3 2	21.90
10 a. m., Dec. 2, to 9 a. m., Dec. 3 <sup>1</sup> .....	2 9	15.50
10 a. m., Dec. 4, to 7 p. m., Dec. 5 <sup>1</sup> .....	1 0	25.00

<sup>1</sup> The low germination in the tests that were started at 10 a. m. is believed to be due to the cloudy winter weather which is unfavorable for mildew development and which was prevailing at this time.

The diurnal cycle of germinability of conidia appears to be a direct or indirect result of the alternation of light and darkness to which the mildewed plants are normally subjected in nature. When mildewed plants were grown in control chambers in a 12-hour day where light was the only environmental factor known to vary throughout the day, a definite diurnal cycle of conidial germination was apparent, as previously noted.

#### EFFECT OF LIGHT ON ELONGATION OF HYPHAE AND FORMATION OF APPRESSORIA

Plants grown in the greenhouse were inoculated and placed in the environmental control chambers at about 24° C. and 60 percent relative humidity for a 12-hour day. Samples of leaves were removed from the plants at about 6-hour intervals, and the lengths of hyphae

were measured and the appressoria counted in individual fungus colonies. The average size of the colonies and the number of appressoria were determined, after staining, from measurements and counts on each of 5 to 10 colonies on each of 4 to 10 leaflets removed at each time of sampling. The rate of growth of mildew colonies was followed in this way in three tests. These tests gave similar results. The data of one test are given in table 3. The length of hyphae increased at a fairly uniform rate in light and in darkness, but appressoria were formed almost entirely in the light only.

When inoculated plants were exposed to 6-hour periods of alternating light and darkness the rate of increase of colony size and number of appressoria was less than on plants in a 12-hour day, especially at the beginning of the test, and appressoria were formed in about equal numbers in light and in darkness.

TABLE 3.—*Elongation of the hyphae of Erysiphe polygoni and formation of appressoria in light and in darkness, Madison, Wis., 1934*

Time of sampling	Period between inoculation and sampling	Light condition of interval preceding measurement or count	Growth of fungus on leaves exposed to alternate 12-hour periods of light and darkness	
			Average length of mycelium in colony	Average number of appressoria per colony
	Hours		$\mu$	
12 p m., Jan 20.....	16	Dark.....	83	1 0
7 a. m., Jan. 21.....	23	.....do.....	232	1 0
12 m., Jan 21.....	28	Light.....	462	2 1
6 p. m., Jan 21.....	34	.....do.....	683	4 2
12 p m., Jan 21.....	40	Dark.....	1, 140	4 5
7 a m., Jan 22.....	47	.....do.....	1, 780	5 0
12 m., Jan 22.....	25	Light.....	2, 465	9 2

<sup>1</sup> Primary appressorium from the germ tube.

In determining the effect of light on the rate of colony growth by the previous method, growth was measured as the total increase in the length of hyphae per colony on the sampled leaves, but the rate of elongation of individual hyphae tips could not be followed by that method. In another test young recently inoculated leaves were excised from plants and floated on water in dishes in the control chambers. Several individual hyphal tips were selected, their positions were marked by means of drawing at the beginning of the test, and the subsequent growth measured at intervals thereafter. In measurements of 30 hyphal tips at about 3-hour intervals throughout the 24 hours of the day, the hyphae grew at an average rate of about 20 $\mu$  per hour, and no significant difference in the rate of growth in light and in darkness could be detected.

The total length of hyphae and the number of appressoria per colony were determined in two experiments in which the host plants placed in the control chambers were given light exposures of various lengths. The results of one experiment are given in table 4. At 32 hours after inoculation the fungus colonies on the plants held in continuous darkness after inoculation were smaller than those exposed to even 4 hours of light per day, and at 48 hours after inoculation the differences were very large. At 48 hours after inoculation the size of e colonies (the total length of hyphae) increased with the length of

light exposure from  $699\mu$  per colony in darkness to  $1,880\mu$  in a 12-hour day, and then decreased with increased light to  $1,500\mu$  in continuous light. The number of appressoria per colony showed the same general trend, increasing from 3.3 in continuous dark to 10.4 per colony in a 12-hour day, and falling to 2.1 in continuous light. The average length of hyphae per appressorium showed more clearly than did the size of the colonies or the number of appressoria per colony the effect of the length of day on mildew development. For example, on the plants in continuous darkness, the mildew colonies, which never reached the sporulation stage, developed an average of  $212\mu$  of hyphae per appressorium. On plants in 4, 8, 12, and 16 hours of light per day, the colonies, which later showed a well-marked diurnal cycle of sporulation, had from  $165\mu$  to  $190\mu$  of hyphae per appressorium. In contrast, the colonies developing on plants in light exposures of 20 and 24 hours per day, which showed a greatly disturbed cycle of sporulation, as described later, had  $624\mu$  and  $714\mu$  of hyphae per appressorium, respectively.

TABLE 4.—Relation of length of day to mycelial elongation and appressorium formation of *Erysiphe polygoni*

Light condition per day	Growth 32 hours after inoculation			Growth 48 hours after inoculation		
	Average total length of hyphae per colony	Average number of appressoria per colony	Length of hyphae per appressorium	Average total length of hyphae per colony	Average number of appressoria per colony	Length of hyphae per appressorium
Continuous darkness.....	$\mu$ 270	1.68	$\mu$ 160	$\mu$ 699	3.3	$\mu$ 212
4 hours light.....	616	1.78	346	1,070	6.4	167
8 hours light.....	625	1.92	326	1,620	9.8	165
12 hours light.....	644	2.17	297	1,880	10.4	180
16 hours light.....	609	2.02	302	1,678	8.8	190
20 hours light.....	571	2.40	238	1,620	2.6	624
Continuous light.....	593	1.62	366	1,500	2.1	714

The increase in the size of the colonies as the period of exposure to light was increased from 0 to 12 hours per day is interpreted by the writer as mainly due to the nutritional conditions of the fungus, the increased exposure to light resulting in a greater supply of carbohydrates in the parasitized leaves. That light is in itself not necessary or even very stimulatory for mildew growth has been shown by dish-culture experiments in which some inoculated leaflets floating on sugar solutions were exposed to the natural light of the laboratory and others were exposed to total darkness. The mildew development was luxuriant and similar in both cases, although slightly better on the leaves exposed to the light. The decrease in colony size as the light exposure was increased from 12 to 24 hours per day may have been due in part to the few appressoria, and hence also the few haustoria through which the fungus could draw its food supply.

The small number of appressoria per colony in continuous darkness, while much less than for colonies in alternating light and darkness of 4 to 16 hours of light per day, is not greatly less when the size of the colonies is taken into consideration. The small number of appressoria per colony, however, both in absolute numbers and in proportion to the size of the colony in 20 and 24 hours of light per day appears at present to be a manifestation of a disturbed diurnal cycle of the fungus. It is interesting that while in a 12-hour day appressoria were formed almost entirely during the light period, yet in 20 or 24 hours of

light per day there were fewer appressoria formed per colony or per unit length of mycelium than in continuous darkness. Certainly, normal appressorium formation can hardly be a direct response to light, neither does it appear to be favored by darkness; it must be dependent on the alternation of light and darkness, which is necessary for the manifestation of the normal cycle of the mildew fungus.

#### FORMATION AND ABSTRICTION OF CONIDIA

Sporulation of clover powdery mildew begins about 5 days after a susceptible plant has been inoculated. The initiation of the conidiophores has not been followed by the writer, but the changes undergone by the mature conidiophores throughout the day have been observed on plants growing in the field, in the greenhouse, and in the control chamber. To observe the conidiophores, leaflets with young vigorous mildew colonies were folded of a slide with the undisturbed conidiophores on the upper surface on the leaf projecting beyond the

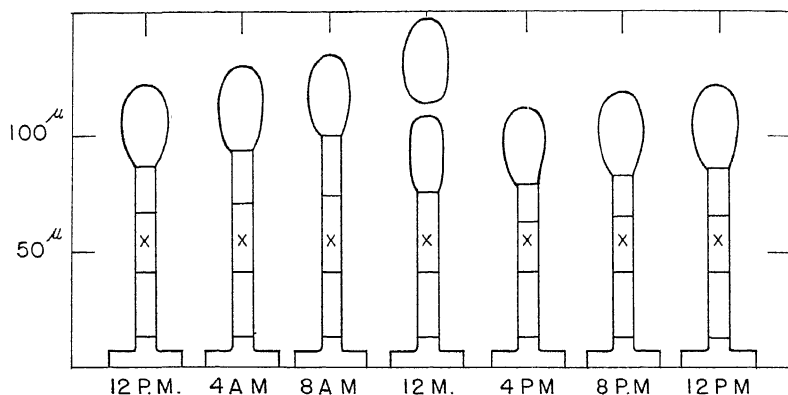


FIGURE 1.—Diagrammatic representation of the abstriction of conidia and of the changes in the conidiophore throughout the day, as observed on mildewed clover plants in the field at Madison, Wis., September 4, 1933. The generative cell (x) is the third cell from below.

edge of the fold, where they could be examined with the low- or high-power objectives of the microscope. The diurnal changes observed in the conidiophores on field plants on September 4, 1933 (13-hour day, sun rose at 5:25 a. m. and set at 6:28 p. m.), are illustrated in figure 1, and are quite typical of numerous observations made earlier and later in the season outdoors and in the greenhouse. The conidia were abstricted between 8 a. m. and 12 m. The abstricted conidium appears to remain passively attached to the conidiophore until it is dislodged by air currents or by disturbances of the leaves. After the abstriction of the conidium, the terminal cell of the conidiophore gradually increased in diameter (mostly during the light portion of the day) to form a new conidium which was abstricted the following morning. Thus each normally functioning conidiophore abstricts one conidium per day. The generative cell increased gradually in length and divided into two cells about 4 p. m. by a septum nearer its distal end. The basal cell showed no change in its gross morphology throughout the day.

Variations in this process are caused by seasonal and daily weather changes, but these have not been studied. However, on all days on



which the maturation of conidia was followed in the field in 1933 the conidia were not abstricted at 8 a. m. and were completely abstricted at 12 m. In the control chamber in a 12-hour day conidia were all abstricted 4 hours after the beginning of the light period.

The diurnal cycle of the abstriction of conidia is extremely definite and easily observed on vigorous mildew colonies and during clear weather. The developmental changes in the conidiophore are gradual, but the abstriction of conidia and the division of the generative cell are very specific processes which are generally complete on 90 percent of the conidiophores within the period of 2 hours for each process. In dull cloudy weather the diurnal cycle of the maturation of conidia is much less regular.

The diurnal cycle of the formation of conidia, like the diurnal cycle of conidial germinability, is dependent on the alternate light and darkness of the normal day. In the environmental control chambers with day lengths of 4, 8, 12, 16, and 20 hours, there was definite diurnal cycle of conidium formation, but in continuous darkness, in 2 hours of light per day, 22 hours of light per day, and continuous light no cycle of conidium formation was apparent. When plants subjected to a 12-hour day were given their daily light exposure from 8 p. m. to 8 a. m. instead of from 8 a. m. to 8 p. m., as was usually done, conidium formation followed a cycle, the various stages of which occurred 12 hours later than in the normal 12-hour day.

#### SPORE DISSEMINATION

Determinations of spore dissemination were made in the field during June, July, August, and September 1933, by counting the conidia caught during known intervals of time on slides placed in a field plot of mildewed clover. Three slides were placed on the ground at three different locations in the field and were changed at about 4-hour intervals during the day and less frequently at night. The determinations covered six series of observations of 1 day or more each, totaling 15 days in all. The results for 1 day or more in each of five of these series are given in table 5. In all cases the greatest liberation of spores occurred during the day, the maximum dissemination occurring about 12 m. This is a little later than the time of maximum spore maturation.

TABLE 5—*Spore dissemination from mildewed clover plants in the field as determined by number of conidia caught at different times throughout the day and night*

Series	Period in which conidia were caught	Average number of conidia <sup>1</sup>	Series	Period in which conidia were caught	Average number of conidia <sup>1</sup>
No. 1	2 a. m. to 6 a. m., June 16.....	0.05	No. 4	7 p. m., July 15, to 10 a. m., July 16.....	1.6
	6 a. m. to 10 a. m., June 16.....	6.4		10 a. m. to 1 p. m., July 16.....	47.4
	10 a. m. to 2 p. m., June 16.....	15.6		1 p. m. to 3 p. m., July 16.....	44.4
	2 p. m. to 6 p. m., June 16.....	3.5		3 p. m. to 6 p. m., July 16.....	25.4
	6 p. m. to 10 p. m., June 16.....	.11		6 p. m. to 8 p. m., July 16.....	9.4
	10 p. m., June 16, to 8 a. m., June 17.....	16		8 p. m. to 10 p. m., July 16.....	3.1
No. 2	2 a. m. to 4 a. m., June 28.....	5.0	No. 5	10 p. m., July 16, to 6 a. m., July 17.....	1.6
	4 a. m. to 10 a. m., June 28.....	12.0		9 p. m., Sept. 6, to 6 a. m., Sept. 7.....	.7
	10 a. m. to 12 m., June 28.....	39.1		6 p. m. to 12 m., Sept. 7.....	8.8
	12 m. to 2 p. m., June 28.....	43.4		12 m. to 4 p. m., Sept. 7.....	12.0
	2 p. m. to 4 p. m., June 28.....	61.2		4 p. m. to 7 p. m., Sept. 7.....	5.6
	4 p. m. to 6 p. m., June 28.....	34.3		7 p. m. to 11 p. m., Sept. 7.....	1.4
No. 3	8 p. m., July 5, to 8 a. m., July 6.....	11.2		11 p. m. to 7 a. m., Sept. 8.....	.7
	8 a. m. to 12 m., July 6.....	70.0			
	12 m. to 4 p. m., July 6.....	58.7			
	4 p. m. to 8 p. m., July 6.....	41.8			
	8 p. m., July 6, to 8 a. m., July 7.....	21.9			

<sup>1</sup> Caught per hour per square centimeter of slide.

## EFFECT OF TIME OF INOCULATION UPON INFECTION

Field plants, potted plants, excised leaves with their petioles in water, and excised leaflets floating on sucrose solution have been used extensively in inoculation experiments. For the tests of the relative success of inoculations at different times during the day, most of the inoculations were performed by dusting healthy leaves with spores from field or greenhouse plants, but in some cases selected leaflets were inoculated by touching them with a heavily mildewed leaf. The severity of mildew resulting in 4 to 7 days was estimated by the method considered most satisfactory for the particular test. In some cases the individual leaflets were rated for their relative mildew severity and the values averaged for each unit of a test. With mass inoculations on potted plants the mildew was estimated for each pot as a unit. With inoculations on individual leaves on field plants, one leaflet was left uninoculated as a control on which to estimate contamination.

The results of these tests are given in table 6, where the values for mildew development in different tests are brought to the same basis by considering the mildew development on the optimum unit of each test as 10 and expressing the results of inoculations made at other periods in relation to this maximum infection.

TABLE 6.—Effect on mildew development of the period of day when inoculations were made

Date of test	Location	Host material	Relative amount of mildew resulting from inoculations at <sup>1</sup> —					
			1 to 5 a. m.	5 to 9 a. m.	9 a. m. to 1 p. m.	1 to 5 p. m.	5 to 9 p. m.	9 p. m. to 1 a. m.
1932								
June 24	Field	Excised leaves		0.5		10.0	6.6	0.7
July 2	do.	do.				7.5	10.0	4.5
Do.	do.	do.		3.3	2.7	4.9	3.0	5.7
July 9	do.	do.	{			8.5	10.0	3.9
				2.9		7.2	4.7	2.9
1933								
June 5	do.	Field plants		6.4		1.2	10.0	1.0
June 12	do.	do.				6.2	10.0	10.0
June 14	do.	do.				10.0	5.2	7.2
July 6	do.	Potted plants				10.0	10.0	4.0
Aug. 25	do.	Field plants	0.4			5.1	10.0	1.1
Do	do.	Potted plants				10.0	5.2	5.8
Sept. 1	do.	do.	3.2	7.2	9.8	8.0	10.0	6.4
Sept. 18	Field and greenhouse <sup>1</sup>	do.	1.5	8.5	10.0	7.5	1.0	1.5
Sept. 22	do.	do.	1.8	4.1	10.0	5.3	1.8	2.3
Oct. 5	Greenhouse	do.	1.4	7.5	5.6	10.0	2.6	1.4
Average			1.6	5.0	7.6	7.4	6.6	3.9

<sup>1</sup> 10 represents maximum infection.

<sup>2</sup> Average of 1 series in field and 2 in greenhouse.

The mildew development resulting in the different tests varied considerably. Some infection resulted from inoculations made at any time during the normal day, but inoculations made during the period of daylight were most successful. The success of inoculations made during the hottest and driest periods of sunny days illustrates the extreme tolerance of this fungus to low humidity.

In the previously reported tests of mildew infection throughout the light and dark portions of the day, the evaluation of the causes for differences in infection resulting from inoculations at different periods was difficult because of the simultaneous variation in the fungus, the host, and the environment. In a few experiments a more adequate separation of the influence of light from the other factors was obtained by inoculating two groups of plants simultaneously with the same inoculum and incubating them in a similar environment, except that one group was placed in the natural light and the other was placed in darkness for the first few hours or from the time of inoculation until nightfall, and afterward all were exposed to the same environment. The results of several such tests are given in table 7. In most cases heavier infection resulted on plants incubated in the light, but generally the difference was small and in some cases no difference was apparent.

TABLE 7.—Effect of light on mildew infection in 2 groups of plants inoculated simultaneously<sup>1</sup>

Time of inoculation	Host material	Additional environ- mental condition of host material	Relative mildew infection resulting in group kept in—	
			Light	Darkness
1932				
2 p. m., Nov. 19	Potted plants		10.0	3.0
Do.	do.	Humid bell jars	3.0	1.0
1933				
2 p. m., Feb. 2	Excised leaves	Petri dishes	4.2	2.5
2 p. m., Feb. 6	do.	do.	3.1	2.0
11 a. m., Feb. 21	do.	do.	3.1	1.8
12 m., Mar. 2	do.	do.	4.2	2.5
Mar. 30 <sup>2</sup>	Potted plants	do.	9.5	7.5
Apr. 15 <sup>3</sup>	do.	do.	9.1	9.1
5 p. m., Apr. 17	do.	do.	9.0	9.0
5 p. m., Apr. 20	do.	do.	9.0	9.0

<sup>1</sup> Tests were made in the greenhouse.

<sup>2</sup> Average of 2 inoculations, 1 at 1 p. m. and the other at 5 p. m.

<sup>3</sup> 3 inoculations, 1 at 10 a. m., 1 at 2 p. m., and 1 at 5 p. m., did not give apparent differences due to light

The writer believes that infection (the establishment of a nutritional relation between host and parasite) of red clover with *Erysiphe polygoni* normally occurs in greatest abundance during the light portion of the day, and this belief is supported by the following facts: (1) Conidia were disseminated in greatest abundance during the light portion of the day; (2) germination of conidia was favored by light; (3) formation of appressoria was favored by light; (4) conidia taken from diseased plants during the light portion of the day germinated more readily than those taken at night; (5) the closure of clover leaves at night by the coming together of the two lateral leaflets and the movement of the center leaflet to a vertical position would reduce the chances of air-borne conidia falling on the leaves; (6) inoculations made during the day were more successful than those made at night; and (7) inoculations made in the light were more successful than inoculations made in darkness.

The relative importance of the various factors favoring infection during the light portion of the day cannot be determined from the present results, but all probably play a part. The dissemination of conidia during the light portion of the day and their rapid germination would be important. The fact that conidial germination is

greatly stimulated by host leaves would reduce the importance of the effect of light on germination and appressorium formation and of the diurnal cycle of conidial germinability as factors determining when infection might take place. The determinations of the dissemination of conidia and of conidial germination on dry slides in the field indicate that infection occurs principally in the latter part of the normal light period.

### TRANSPIRATION OF MILDEWED AND OF HEALTHY CLOVER LEAVES

Leaves were excised from mildewed and from healthy greenhouse plants of the same age and general history except for inoculation, and were placed with their petioles in flasks of water (four leaves per flask), which were then tightly stoppered with cotton. The transpiration of five flasks of mildewed leaves and of five flasks of healthy leaves was followed under greenhouse conditions, and also that of five flasks of mildewed leaves and five flasks of healthy leaves in the constant light, temperature, and humidity of the control chamber. Weighings were made to one one-hundredth of a gram at intervals of about 3 hours and measurements of water loss were continued for 29 hours. As the ratio of dry weight to leaf area was not significantly different for healthy and mildewed leaves ( $0.434 \pm 0.0095$  g per square decimeter for healthy and  $0.430 \pm 0.0078$  g per square decimeter for mildewed leaves) the results are expressed on a dry-weight basis. The average results of this test (table 8) show that in continuous light the transpiration of mildewed leaves was about the same as the transpiration of healthy leaves, and neither showed any diurnal variation. In the greenhouse, however, the transpiration of both diseased and healthy leaves showed the expected general variation between day and night, but while the transpiration of diseased leaves was similar to that of healthy leaves during the day, the transpiration of mildewed leaves was much greater at night. Four other tests in less detail gave similar results. In one test in which transpiration at  $15^{\circ}$  C. was compared with that at  $30^{\circ}$  the increased transpiration at night due to mildew infection was much greater at  $15^{\circ}$  than at  $30^{\circ}$ .

TABLE 8.—*Effect of mildew infection on the transpiration of clover leaves throughout the day, Dec. 11–12, 1933, at Madison, Wis.*

Time interval	Effect of infection on transpiration of clover leaves					
	In the greenhouse			In continuous light		
	Water lost per gram dry weight of leaves per hour		Increase or decrease in transpiration associated with mildew infection	Water lost per gram dry weight of leaves per hour		Increase or decrease in transpiration associated with mildew infection
	Healthy leaves	Mildewed leaves		Healthy leaves	Mildewed leaves	
	Grams	Grams		Grams	Grams	
11:15 a. m. to 12:30 p. m.-----	5.78	5.60	-3.11	2.01	1.71	-14.9
12:30 p. m. to 3 p. m.-----	6.19	6.23	+ .65	2.55	2.61	+2.35
3:30 p. m. to 4:50 p. m.-----	3.38	4.11	+21.6	2.36	2.16	-8.47
4:50 p. m. to 7 p. m.-----	1.06	1.71	+61.3	2.48	2.18	-12.1
7 p. m. to 9 p. m.-----	.67	1.13	+68.86	2.58	2.71	+5.04
9 p. m. to 11:30 p. m.-----	.64	1.17	+82.8			
11:30 p. m. to 7:30 a. m.-----	.84	1.51	+79.76	2.19	2.14	-2.28
7:30 a. m. to 10:25 a. m.-----	3.17	3.07	-3.15	2.14	2.25	+5.14
10:25 a. m. to 12:40 p. m.-----	4.81	4.75	-1.04			
12:40 p. m. to 3:05 p. m.-----	5.91	5.89	-1.33	1.72	1.81	+5.23

The resemblance between the effect of mildew on transpiration, as observed by the writer, and the effect of bordeaux mixture on transpiration, as observed by Duggar and Bonns<sup>2</sup> and others, is interesting. In both cases the effect is generally to greatly increase the transpiration of leaves during the night and to slightly decrease it during the light portion of the day. Because other phases of the development of clover mildew have been clearly shown to undergo a diurnal cycle as already described, the effect of mildew on transpiration may also possibly be due to a diurnal cycle of the fungus, but the work of Graf-Marín<sup>3</sup> would indicate that the cause of the day-and-night variation in the effect of mildew on transpiration was due to the smaller opening of the stomata of diseased leaves during the light portion of the day.

#### STUDIES OF OTHER POWDERY MILDEWS

*Erysiphe polygoni* was abundant on delphinium and on cabbage in the greenhouse in the fall and winter of 1933. These two forms of *E. polygoni* can be readily distinguished from the form on red clover and from each other by the size of the conidiophores and conidia, and by cross-inoculation tests. To the extent that they have been studied—namely, in the germinative capacity of the conidia and the development of the conidiophores—they show the same diurnal cycle as *E. polygoni* on red clover. Since this cycle was apparent on diseased plants which were exposed to constant temperature and humidity but to a 12-hour day, it appears that, as with *E. polygoni* on the red clover, the cause of the diurnal cycle of the fungus on cabbage and on delphinium is the alternation of light and darkness.

*Erysiphe graminis* DC., which is sharply different morphologically from *E. polygoni*, was also studied with respect to its development throughout the day and night. The basal cell of the conidiophore of *E. graminis* is decidedly swollen, and the transition from immature to mature conidia is gradual through several cells of the chain of conidia. For this reason it is difficult to trace the development of the conidiophore of this parasite in its relation to day and night. The results of several tests to determine whether *E. graminis* on barley showed a diurnal cycle in the germinative capacity of its conidia and in its ability to cause infection have shown no evidence of a definite variation between day and night. The germination of conidia was stimulated by light, however, as was the germination of the conidia of the other Erysiphaceae studied.

#### DISCUSSION

The writer's present conception of the diurnal cycle of *Erysiphe polygoni* is that certain phases of fungus development tend to occur during the light portion of the day and in some cases at a specific time of the day. Practically all the conidia formed during the 24 hours of the day are matured during the latter part of the morning (8 a. m. to 12 m.). The division of the generative cell occurs during a very limited period in the late afternoon and early evening. The

<sup>2</sup> DUGGAR, B. M., and BONNS, W. W. THE EFFECT OF BORDEAUX MIXTURE ON THE RATE OF TRANSPIRATION. *Ann. Mo. Bot. Gard.* 5: 153-176, illus. 1918.

<sup>3</sup> GRAF-MARÍN, A. STUDIES ON POWDERY MILDEW OF CEREALS. N. Y. (Cornell) *Agr. Expt. Sta. Mem.* 157, 48 pp., illus. 1934.

dissemination of conidia, on the other hand, being presumably a passive process but dependent on the maturation of the conidia, on wind, and on the dryness of the leaf and fungus surface, is more or less continuous throughout the light portion of the day, but is less at night, perhaps because the humidity is higher then and there is less wind. Appressoria are normally formed during the light portion of the day, and they may be formed throughout the light period, but they form very readily in darkness under certain experimental conditions.

The greater activity of *Erysiphe polygoni* during the light portion of the day, and its ability to sporulate and cause infection under conditions of relatively low atmospheric humidity are in sharp contrast to certain downy mildews described by Weston,<sup>4</sup> in which the greatest activity of the fungus is in the dark and humid portion of the day. The basic cause of the diurnal cycle of an obligate parasite is difficult to determine, but the regular alternation of light and darkness appears necessary for the expression of the diurnal cycle of *E. polygoni*. The length of either the light or the dark portion of the day is of less importance, as indicated by the expression of the cycle in photoperiods of 4 to 20 hours. The great stimulatory effect of light on conidial germination on glass slides would indicate that the cycle might be due in large part to the direct effect of light on the fungus, but the luxuriant development of mildew on leaves in total darkness but floating on sucrose solution would not support this idea.

#### SUMMARY

Conidia of clover mildew (*Erysiphe polygoni*) germinated best when removed from diseased plants in the afternoon. With the onset of darkness the germinability of the conidia gradually decreased, reaching a minimum in the early morning but again rising with the onset of daylight. Attempts to increase the germination of conidia in the low phase of the germinability cycle have been successful only by exposing them to light or by placing them on host leaves.

The elongation of the hyphae of clover mildew continued at a fairly uniform rate throughout the day and night, but appressoria were formed principally during the light.

The generative cell of the conidiophore divided once each day in the late afternoon to form a new proximal generative cell and a distal daughter cell. One conidium was matured during the late morning on each conidiophore.

Conidia were caught on spore-trap slides in greatest abundance about noon, but there was a continuous dissemination of conidia throughout the middle and latter part of the daylight period, and few were caught at night.

The diurnal cycle manifest in the maturation of conidia and in the division of the generative cell was clearly evident on mildewed plants growing in 4, 8, 12, 16, and 20 hours of light per 24 hours, but in longer or shorter light exposures per day the conidiophores did not develop normally and the diurnal cycle was less evident or was no longer apparent.

Inoculations with clover mildew during the light portion of the day were more successful than inoculations made at night.

<sup>4</sup> WESTON, W. H. PRODUCTION AND DISPERSAL OF CONIDIA IN THE PHILIPPINE SCLEROSPORAS OF MAIZE. Jour. Agr. Research 23: 239-278, illus. 1923.

The transpiration of mildewed leaves was greater than that of healthy leaves at night and was about equal to or slightly less than that of healthy leaves during the day.

The diurnal cycles manifest in conidial formation, conidial germination, and appressorium formation were evident when temperature and humidity were constant, with plants growing in a 12-hour day, but these cycles, as well as that of transpiration, were eliminated when the test plants were grown in continuous light. It is concluded that the alternating light and dark of the normal day are responsible for the cyclic manifestations of *Erysiphe polygoni* described here.

So far as they were studied, the forms of *Erysiphe polygoni* on red clover, delphinium, and cabbage showed similar diurnal cycles, but no diurnal cycle of development was established with *E. graminis* on barley.





# HOST RANGE AND PHYSIOLOGIC SPECIALIZATION OF RED CLOVER POWDERY MILDEW, *ERYSIPHE POLYGONI*<sup>1</sup>

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## INTRODUCTION

Since 1902, Neger (7)<sup>4</sup> and numerous other workers have shown that the morphologic species *Erysiphe polygoni* DC., parasitizing, according to Salmon (8), some 357 species in 157 genera, consists of a large number of physiologic races. Single races, may, though rarely, infect host species of different genera within a single plant family or may infect several species within a genus or, more rarely, may be limited to a single host species. Blumer (1), however, has divided the *Erysiphe polygoni* of Salmon into several species on the basis of the perfect stage. Of these, *E. martii* Lev., confined to species of the Leguminosae including red clover (*Trifolium pratense* L.), consists of several physiologic races (2).

The purpose of the present investigations was to add to the knowledge of the identity of the powdery mildew of red clover in the United States by studies of the host range and possible physiologic forms of the causative organisms.

## HOST RANGE

The host range of the powdery mildew parasitizing red clover has been extensively investigated, but with extremely variable results. All investigators except Blumer (1), however, agree that the form found on red clover is limited to the host genus *Trifolium*. Salmon (9), Mains (5), and Blumer (1) used entire plants as hosts. Hammerlund (2) and Searle (10) used both entire plants and excised leaves in Petri-dish moist chambers. In a previous study the writer used entire plants in the field and the greenhouse, and excised leaves on sugar solution in dish cultures (11).

In the greenhouse and in dish-culture tests leaves of five plants of each host species were inoculated in most cases, and infection was considered positive if one or more plants of a species were infected. In the field a record was kept of the natural mildew infection of plants in a plot containing a number of *Trifolium* species adjacent to a plot of heavily mildewed red clover. That the mildew occurring on these *Trifolium* species in the field resulted from infection with conidia from red clover is not certain but appears very likely.

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<sup>2</sup> The writer wishes to express his indebtedness to E. B. Mains, formerly agent, Division of Cereal Crops and Diseases, and to A. J. Pieters, principal agronomist, Division of Forage Crops and Diseases, Bureau of Plant Industry, for assistance during the course of this work.

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<sup>4</sup> Reference is made by number (italic) to Literature Cited, p. 664.

The greenhouse inoculations were performed by dusting young plants of the *Trifolium* species with conidia from mildewed red clover. No precautions against contamination were taken, but, as red clover powdery mildew was the only form of *Erysiphe polygoni* in the greenhouse at the time of these tests, contamination was unlikely.

In the dish-culture tests, leaflets were excised from mildew-free greenhouse plants, inoculated by touching their upper surfaces with a heavily sporulating mildewed leaf of red clover, and floated on 10-percent sucrose solution in Syracuse watch glasses in the diffuse light of the laboratory at about 20° C. Inoculated leaves were examined for infection about 10 days after inoculation.

In table 1 the *Trifolium* species tested are listed in the order of their apparent susceptibility, and the summarized results of other investigators are tabulated for comparison. It is difficult to express quantitatively the relative susceptibility of these species, but it may be said that *T. lupinaster* L. was almost if not quite as susceptible as *T. pratense*, that *T. hybridum* L. was much less susceptible, and that such species as *T. repens* L. and *T. incarnatum* L. were definitely resistant although sporulation of *Erysiphe polygoni* on excised leaflets of these species was observed in dish cultures. Each species tested in dish culture was inoculated separately with physiologic forms 1 and 2 of clover mildew, described later, but, since none of these species other than *T. pratense* showed any difference in susceptibility to the two forms of mildew, the results are not separately listed.

Leaflets of *Trifolium pratense* were inoculated in dish culture with conidia produced on each of the species listed as susceptible. Reciprocal inoculation was successful from all species but *T. incarnatum*.

The results given in table 1 emphasize the great variation in the results of different workers. In contrast to 1 susceptible species out of 8 tested by Salmon (9), 1 susceptible species out of the 3 tested by Searle (10), 1 susceptible species out of the 7 tested by Mains (5), 2 susceptible species out of the 8 tested by Hammerlund (2), and 4 susceptible species out of the 20 tested by Blumer (1), the writer found 14 species susceptible in varying degrees out of a total of 20 species tested. The reason for this extreme variation in the results of different investigators is unknown, but the following possible reasons are suggested:

TABLE 1.—Infection of *Trifolium* species with conidia of *Erysiphe polygoni* from *T. pratense*<sup>1</sup>

Host	Reaction of <i>Trifolium</i> spp. to <i>Erysiphe polygoni</i> according to—								
	Salmon	Searle	Mains	Hammerlund	Blumer <sup>1</sup>	Yarwood, 1931, La Fayette, Ind.			
						Field tests	Green-house tests	Dish cultures <sup>2</sup>	
								Plants inoculated	Plants infected
								Number	Number
<i>T. pratense</i> L.....	+	+	+	+	+	+	+	5	5
<i>T. lupinaster</i> L.....	+	+	+	+	+	+	+	4	4
<i>T. carolinianum</i> Michx.	+	+	+	+	+	+	+	2	2
<i>T. striatum</i> L.....	+	+	+	+	+	+	+	3	3
<i>T. reflexum</i> L.....	+	+	—	+	+	+	+	3	2
<i>T. angustifolium</i> L.....	+	+	+	+	+	+	+	5	5
<i>T. panormitanum</i> Presl	+	+	+	+	+	+	+	5	4
<i>T. hybridum</i> L.....	—	—	—	—	+	+	+	5	4
<i>T. squarrosum</i> L.....	+	+	+	+	+	+	+	4	3
<i>T. resupinatum</i> L.....	+	+	+	+	+	+	+	5	5
<i>T. subterraneum</i> L.....	+	+	+	+	+	+	+	5	4
<i>T. fragiferum</i> L.....	+	+	+	+	+	+	+	5	5
<i>T. repens</i> L.....	—	—	—	—	+	+	+	5	4
<i>T. incarnatum</i> L.....	—	—	—	—	+	+	+	5	4
<i>T. alexandrinum</i> L.....	—	—	—	—	+	+	+	5	4
<i>T. dubium</i> Sibth.	—	—	—	—	+	+	+	5	4
<i>T. medium</i> L.....	—	—	—	+	+	+	+	5	4
<i>T. pannonicum</i> Jacq.	—	—	—	+	+	+	+	5	4
<i>T. rubens</i> L.....	—	—	—	+	+	+	+	5	4
<i>T. procumbens</i> L.....	—	—	—	+	+	+	+	5	4
<i>T. agrarium</i> L.....	—	—	—	+	+	+	+	5	4
<i>T. badiatum</i> Schreb.	—	—	—	+	+	+	+	5	4
<i>T. montanum</i> L.....	—	—	—	+	+	+	+	5	4
<i>T. thalict Vill.</i>	—	—	—	+	+	+	+	5	4
<i>T. alpinum</i> L.....	—	—	—	+	+	+	+	5	4
<i>T. alpestre</i> L.....	—	—	—	+	+	+	+	5	4
<i>T. ochroleucum</i> Huds.	—	—	—	+	+	+	+	5	4
<i>T. ambiguum</i> Bieb.	—	—	—	+	+	+	+	5	4
<i>T. arvense</i> L.....	—	—	—	+	+	+	+	5	4
<i>T. trichocephalum</i> Bleb.	—	—	—	+	+	+	+	5	4
<i>T. pallescens</i> Schreb.	—	—	—	+	+	+	+	5	4

<sup>1</sup> Plus sign (+) indicates that infection was microscopically visible.<sup>2</sup> This column combines the results of 2 of Blumer's tests (1, table 14). If the results of these 2 tests differed, the positive infection was recorded here.<sup>3</sup> Results of a test of Feb. 6, 1931. This test was repeated at 2 other times with essentially similar results.<sup>4</sup> Weak infection.<sup>5</sup> Weak infection was secured in a previous test.

(1) The use of different physiologic forms of the organism, variations in virulence, and differences in host range; (2) different environmental conditions, especially temperature; (3) vigor of the inoculum, including the germinability of the spores; (4) variation in the host species or inadequate representation of the species; (5) probable geographic variations in strains of *Trifolium*, which make positive results of much greater importance than negative results.

#### PHYSIOLOGIC FORMS

Salmon (9), Searle (10), Blumer (1), Hammerlund (2), and others have shown that the morphologic species *Erysiphe polygoni* of Salmon (10) consists of a number of physiologic races each of which is generally limited to a single genus or species of host plant. While these physiologic races may be indistinguishable morphologically in their perithecial stages, it is likely that most so-called physiologic races or forms of *E. polygoni* can be distinguished from each other by

the morphology of their conidial stages. Homma (3) has shown that there are appreciable morphological differences between the biological forms of *E. graminis* DC. on wheat, hull-less barley, *Poa annua* L., and *Elymus mollis* Trin. The writer has observed that the forms of *E. polygoni* (according to Salmon) on bean, red clover, California poppy, mustard, pea, cowpea, buckwheat, and columbine can be distinguished readily from each other by the gross morphology of their conidiophores and conidia. In the sense that they are clearly distinct morphologically in their conidial stages, these forms occurring on different host genera are probably not true physiologic forms. More-

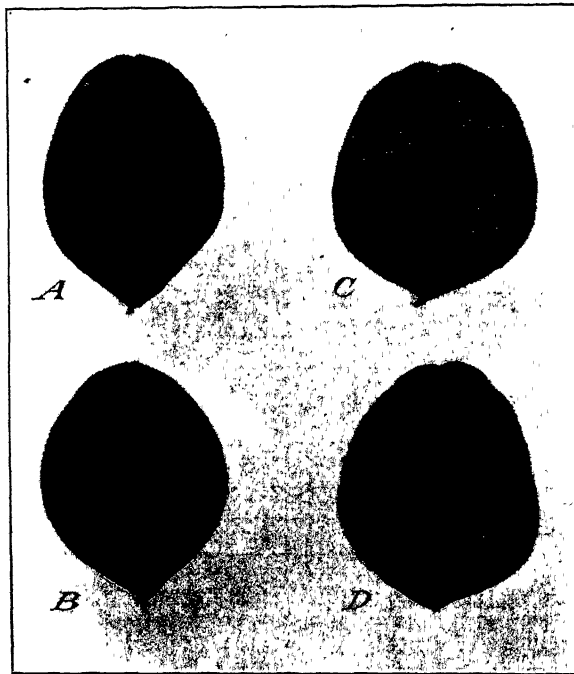


FIGURE 1.—Differentiation of physiologic forms 1 and 2 of clover mildew. A, Plant 23 inoculated with physiologic form 1; B, plant 23 inoculated with physiologic form 2; C, plant 2 inoculated with physiologic form 1; D, plant 2 inoculated with physiologic form 2. Plant 23 is immune from form 1 and susceptible to form 2; plant 2 is equally susceptible to both forms. Photographed 11 days after inoculation.

over, Blumer (1) has distinguished many of these in Europe by the morphology of their perfect stages.

In July 1930 two clones of red clover (22 and 23), previously selected for mildew resistance, became heavily infected with powdery mildew. Healthy leaves of these and other red clover clones were taken from plants which had been protected from infection by dusting with sulphur, the leaflets were washed and inoculated with five collections of red clover powdery mildew in dish culture. Four of the six clones used were equally susceptible to the five mildew collections tested at this time, but clones 22 and 23 were immune (reaction with necrotic spots) (6) to the mildew prevalent in the field and in the greenhouse but were very susceptible to the inoculum taken from the mildew occurring on them. On the basis of this and a later test, two physi-

ologic forms of red clover mildew were distinguished. The mildew which was prevalent in 1930 and 1931 and to which clones 22 and 23 were immune is designated physiologic form 1, and the mildew to which these clones are susceptible is called physiologic form 2. Figure 1 illustrates the mildew reactions by which physiologic forms 1 and 2 of clover mildew were distinguished. Single-spore cultures of forms 1 and 2 of clover mildew were isolated and cross-inoculations repeated with similar results.

In the fall of 1931, a third physiologic form of clover mildew was found on a plant which was very resistant (reaction 1 with necrotic spots) (6) to forms 1 and 2. Except for clearly differentiating it from forms 1 and 2, form 3 has not been studied.

All determinations of physiologic forms of clover mildew were made in dish cultures. In the determination of the physiologic forms of many parasitic fungi, for example the wheat rusts genetically pure lines of the host plants are generally available, and a number of plants of each variety can be inoculated with each of the collections of the parasite to be tested. With such a heterozygous plant as red clover, however, this is not possible, unless the desired differential plants are multiplied by cuttings. This is a slow process, as only a limited number of cuttings can be secured from a given plant at any one time. Also, with a wind-disseminated fungus such as clover mildew, which produces conidia germinating readily in the absence of free moisture, careful greenhouse methods would be necessary to insure against contamination if physiologic forms were to be differentiated on potted plants. These difficulties were readily overcome by the use of dish cultures. If leaflets from the same plant are distributed in different dishes, a single plant may furnish a large number of closely comparable units for a given test, and the same plant may be used repeatedly as a source of mildew-free leaflets. Each unit of a test is enclosed in a glass chamber during the incubation period and thus protected from mildew contamination.

About 800 individual clover plants have been tested for their susceptibility to forms 1 and 2 of clover mildew. No plants susceptible to form 1 and resistant to form 2 have been found, and most of the plants which were more susceptible to form 2 than to form 1 were from crosses involving one or both of the clones on which form 2 was found.

During 1930 and 1931 collections of clover mildew were secured from British Columbia, Canada, and from Maine, New York, Michigan, North Carolina, Kansas, North Dakota, Idaho, and Washington, and seven collections were made in Indiana. These were tested on a series of host plants, including differentials for physiologic forms 1 and 2 of clover mildew, and in most cases for form 3. All collections, except one from Idaho and one from Indiana, corresponded to physiologic form 1 and did not contain forms 2 or 3. In the two exceptions in which form 2 was present, form 1 also may have occurred, since the differentials and methods in use would not determine clearly the presence of form 1 in a mixture of forms 1 and 2. It appears from these limited tests that form 1 is widely prevalent and that forms 2 and 3 are relatively rare. Moreover, since these three forms are similar, they are probably all of the epidemic type which spread so rapidly from the East to the West of the United States from 1921

to 1924.<sup>5</sup> Since powdery mildew of clover was present in the United States previous to 1921,<sup>6</sup> this earlier mildew was probably different from those studied.

Hammerlund (2) secured negative results when red clover was inoculated with the powdery mildew occurring on alsike clover (*Trifolium hybridum*), and Salmon (8), Mains (5), and Hammerlund (2) found alsike clover to be resistant to red clover mildew. However, Blumer (1) secured infection of alsike with the mildew occurring on red clover, and in Idaho (4) reciprocally successful inoculations of the mildews on red and alsike clover have been performed. In the present investigation alsike clover was successfully infected with physiologic forms 1 and 2 from red clover. Powdery mildew on alsike clover at La Fayette, Ind., also was tested on red clover plants and found to correspond to form 1 of red clover mildew.

No differences in the conidiophores and conidia of the three physiologic forms of red clover mildew could be detected, but it is possible that with more refined methods significant variations may be found between the forms. The presence of different forms can be readily determined at any time with certain host plants, but since form 2 was identified by its ability to infect certain plants immune to form 1 the same identical physiologic strains could not be described certainly as the result of future inoculations, except upon the use of identical host plants from which the forms were first described.

#### SUMMARY

Fourteen *Trifolium* species out of 20 tested in the field, in the greenhouse, and in dish cultures showed infection in varying degrees to red clover powdery mildew, *Erysiphe polygoni* DC.

Three physiologic forms of red clover powdery mildew have been distinguished by differences in the reaction of certain red clover clones.

These investigations were greatly facilitated through the use of a method of growing the clover mildew fungus on living excised leaves floating on sugar solution.

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# CHRONIC PROGRESSIVE PNEUMONIA OF SHEEP, WITH PARTICULAR REFERENCE TO ITS ETIOLOGY AND TRANSMISSION<sup>1</sup>

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## INTRODUCTION

The existence of a fatal lung disease of sheep has been recognized among the herds of the northwestern section of the United States, especially in Montana and Oregon, for at least two decades. Since the most prominent symptom of the disease is difficult, or labored, breathing, sheep so affected have been commonly referred to by sheep owners and herdsmen as "lungers."

Marsh (5)<sup>2</sup> made a study of the disease in Montana and published the first report of his findings in 1922. He found the disease to be of the nature of a chronic progressive pneumonia, the lesions in all cases being confined to the thoracic cavity. A disease very similar in character, known as "jagziekte", which has been recognized among the sheep of South Africa for many years, has been reported by Robertson (8), Mitchell (7), Cowdry (1, 2), and others. Because of the striking similarity of the lesions of jagziekte of South Africa and chronic progressive pneumonia of Montana, Cowdry and Marsh (3) made joint comparative studies of the two diseases. They reached the conclusion that they were probably identical.

Chronic progressive pneumonia is unquestionably of considerable economic importance. Marsh (5) estimated that the losses from the disease among Montana sheep range from 2 to 10 percent, varying somewhat in the different herds. The mortality is generally believed to be 100 percent of the animals affected. Observations have indicated that the losses are greater among older animals, but sheep of all ages appear to be susceptible.

The principal objectives of the investigation reported in this paper were to determine the etiological factor or factors involved, the possibility of transmission, and other facts concerning the disease.

## SYMPTOMS AND REPORTED CAUSES OF THE DISEASE

Because of the insidious nature of chronic progressive pneumonia of sheep, it is not known how long the animals are affected before the first clinical symptoms can be observed. The first noticeable symptom is labored breathing after exercise. As the disease progresses there is an acceleration of respiration, dilated nostrils, and flank breathing or pumping, even while the animal is at rest. More or less coughing, with some nasal discharge, has been observed in some cases. In the later stages the affected sheep show marked weakness and emaciation. Breathing becomes more difficult and the disease eventually terminates in death.

<sup>1</sup> Received for publication Jan. 6, 1936, issued June, 1936.

<sup>2</sup> Reference is made by number (italic) to Literature Cited, p. 679.

Several theories as to the cause of this type of chronic pneumonia in sheep and the similar disease jagziekte have been presented by different investigators.

Marsh (6), in his paper on bacteriological studies of chronic progressive pneumonia, reports the frequent isolation from the affected lung tissues of a diphtheroid which he considered an etiological factor, being either associated with, or secondary to, certain other possible predisposing factors. Robertson (8), who was one of the earlier investigators of jagziekte in South African sheep, believed the causative factor in that disease to be of the nature of a protozoan. Mitchell (7), in his studies, pointed to a specific virus as the cause of jagziekte. Cowdry and Marsh (3), in their later studies of the two diseases, reached the conclusion that although these chronic pneumonias, of which the epithelial lesions are particularly characteristic, may be due primarily to a specific cause, the pathological complex as a whole is such as might be caused by a variety of predisposing and exciting factors operating over a period of time.

#### SHEEP USED IN EXPERIMENTS

W. J. Butler, State veterinarian of Montana, at whose request these investigations were first undertaken, cooperated with this Bureau in furnishing, from that State, sheep affected with chronic progressive pneumonia. The sheep were shipped from Montana to the Experiment Station<sup>3</sup> of the Bureau at Bethesda, Md., where most of the transmission experiments were carried on. At various intervals during the course of the investigations the sheep were received in lots of 2, 2, 4, 6, 6, and 10, making a total of 30 sheep received from Montana. Of this number 2 died in transit, 2 were found dead in the pens, and 3 showed only slight pneumonic lesions, leaving 23 to be used in the experiments.

For use in exposure tests, 96 normal sheep were obtained. These were native animals from Maryland. They were in fair condition, and most of them ranged in age from 1 to 1½ years.

#### OUTLINE OF METHOD

Frequent clinical observations were made on the diseased sheep from Montana, and as the animals developed unmistakable symptoms of chronic pneumonia they were killed for study of the gross lesions, histological and bacteriological examinations, and for carrying on transmission experiments.

The sheep were killed by bleeding from the carotid artery and jugular vein. Care was taken not to injure the trachea and thereby permit the inspiration of blood or extraneous contamination. Some of the animals in the advanced stages of the disease were killed shortly after their arrival, and others in which the symptoms were less marked were held for various periods before slaughter. Accordingly, examinations were made of subjects in which the symptoms and lesions varied from slight to those of the advanced type, in which the animals exhibited what was considered to be the last stages of the disease.

At the time gross-lesion examinations were made, small portions of the diseased lung tissue and bronchial and mediastinal lymph glands

<sup>3</sup> This station, now known as Animal Disease Station, has been moved to and made a part of the National Agricultural Research Center, Beltsville, Md.

were placed in fixing solution for later histological examination. Bacteriological examination was made of the lung tissue and associated lymph glands.

Immediately after removal of the lungs from the diseased sheep, the pneumonic areas, including also portions of the lung at the juncture of the diseased and apparently healthy tissue, together with the associated lymph glands, were cultured for aerobic and anaerobic organisms. Aerobic cultures were made by spreading the diseased tissues over the surface of ordinary agar medium and agar medium to which 5 percent of blood serum was added. Anaerobic cultures were made by placing cubes of tissue in depths of 0.1 percent of agar, in Smith fermentation tubes of beef-liver infusion broth containing 1 percent of dextrose, and in tubes of beef infusion broth containing finely divided pieces of beef, the medium being covered with neutral paraffin oil. When micro-organisms were recovered from lung specimens, antigens were prepared from them and agglutination tests and complement-fixation tests were made with blood serum from the host to determine whether specific antibodies were present.

Transmission experiments were made by exposure of healthy sheep to the following materials from diseased sheep: Cultures of organisms from the lung, fresh blood, puslike lung exudate, and emulsified lung tissue, the last-mentioned frequently including portions of the mediastinal lymph gland. Exposure to these materials was made by feeding and by subcutaneous, intravenous, intratracheal, and intrapulmonary inoculations. Contact exposures were also made by placing healthy sheep in pens with the diseased sheep for periods ranging from 6 to 12 months. The exposed sheep were held under observation for varying periods. In the earlier experiments the animals were disposed of in from 3 to 6 months, but in the later experiments most of the sheep were held from 6 to 12 months before slaughter for post-mortem examination. At least 18 of the animals were kept between 11 and 12 months after exposure.

#### DATA OBTAINED FROM SHEEP NATURALLY AFFECTED WITH CHRONIC PROGRESSIVE PNEUMONIA

##### GROSS LESIONS

In making the studies of the gross lesions, it was observed that when the thoracic cavity was opened the lungs did not collapse. There was more or less fluid in the cavity. Extensive pleuritic adhesions were frequently present, and in some cases, as a result of these fibrous proliferations, the lungs appeared constricted or lobulated. There was solidification of the affected lungs, varying in extent from partial to almost complete solidification. The observations of the writers indicated that the solidification of the lung tissue has its beginning most frequently near the borders of the diaphragmatic lobes. The consolidated portion of the lungs was usually of a characteristic grayish color, with prominence of the lung lobules on gross sectioning. Small bronchial concretions were sometimes observed. In some cases a puslike material could be squeezed from the bronchi, bronchioles, and alveoli. The bronchial and mediastinal lymph glands usually were swollen and edematous. In all cases examined the lesions were confined to the thoracic cavity.

## HISTOPATHOLOGICAL FINDINGS

The usual histological picture was that of a chronic catarrhal pneumonia. The walls of the alveoli showed infiltrations of lymphocytes and large mononuclear cells. The cellular exudate in the alveoli consisted of large mononuclear cells and leucocytes occasionally mixed with red cells. The large mononuclear cells in the lumen of the alveoli were frequently found to be greatly swollen and vacuolated (fig. 1, A).

Scattered through the affected lung tissue were frequently seen accumulations of lymphocytes, or lymphoid nodules (fig. 2, A), which have been referred to by Marsh (5) as "tuberclelike nodules." These nodules were found in the peribronchial and also in the interalveolar tissue. In the consolidated portions of the lung tissue most of the alveoli were filled either with the cellular exudate or with mucous plugs. In some cases many of the alveoli and bronchioles were filled with polymorphonuclear leucocytes.

Epithelial proliferations of the alveoli and bronchioles, which are considered characteristic of progressive pneumonia, were observed in practically all cases of the disease examined by the writers (fig. 3). As the disease progressed there was a gradual increase in the fibrous changes in the areas involved, and in the later stages practically complete fibrosis of a considerable portion of the lung tissue was observed in some cases (fig. 4).

The gross pathology and histopathology of chronic progressive pneumonia, as determined by the writers, were essentially the same as the findings of Marsh (5) and Cowdry (3).

## BACTERIOLOGICAL RESULTS

On bacteriological examination, a number of the lung specimens from the sheep naturally affected with chronic progressive pneumonia yielded no growths of micro-organisms, either aerobically or anaerobically. From some specimens there were obtained pure cultures of *Pasteurella ovisseptica*, *Corynebacterium ovis*, fungi, or organisms belonging to the diphtheroid group. Other specimens yielded mixed growths of organisms. In no instance, however, was a positive agglutination or complement-fixation reaction obtainable with antigens made from any of the recovered micro-organisms and the blood serum of the host. Thus, the bacteriological examination of sheep affected with chronic progressive pneumonia gave negative results, no micro-organisms having been found that held any promise of being an etiological factor in the disease.

## RESULTS OF TRANSMISSION EXPERIMENTS WITH HEALTHY SHEEP

Table 1 shows that visible evidence of pneumonia was found in 21 of the 96 healthy sheep exposed. In this group, the lesions varied in extent from rather slight involvement of the lung tissue to well-marked areas of chronic pneumonia.

Subsequent histological examinations of the lung tissue of the 21 sheep revealed that the pneumonia in 7 was very slight, or atypical, in character. In 10, the lesions were found to be typical of verminous pneumonia; Cowdry (2) has called attention to the fact that verminous pneumonia in some cases may simulate the lesions of chronic progressive pneumonia. In the remaining four sheep, nos.

781, 813, 820, and 852, there were well-marked lesions suggestive of chronic progressive pneumonia, and in these cases no nematodes or nematode eggs were found. In view of the fact, therefore, that the

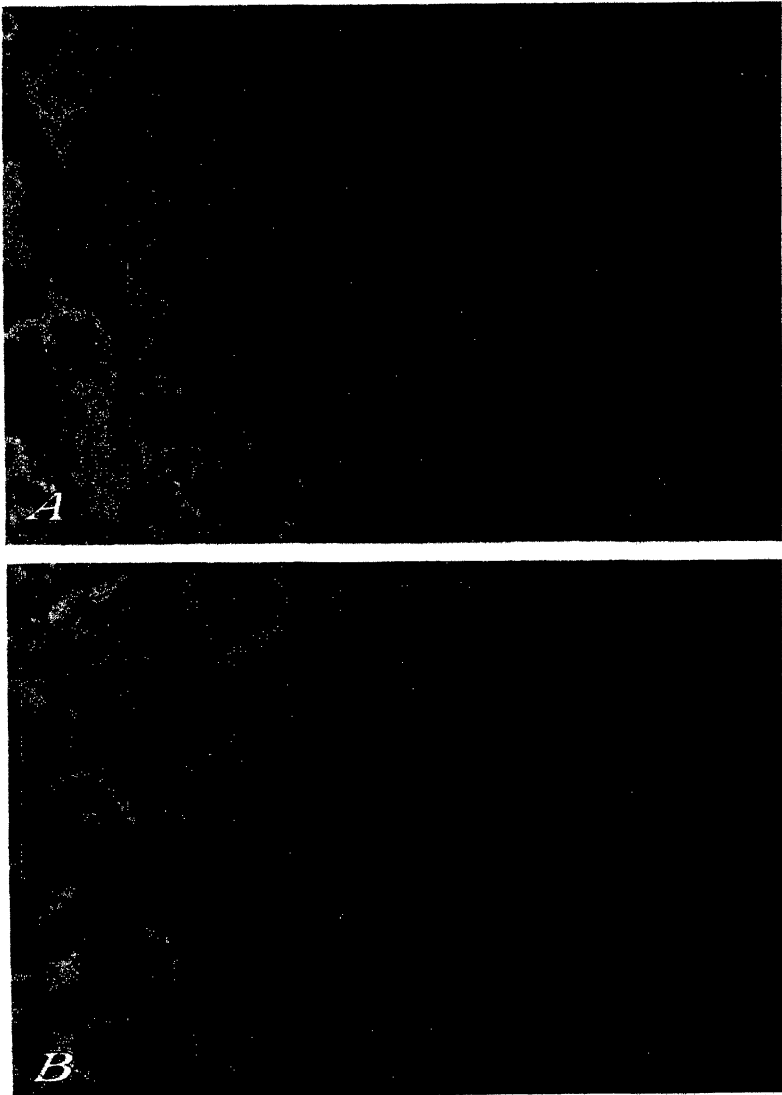


FIGURE 1.—*A*, Section of lung of sheep 693, naturally affected with chronic progressive pneumonia. Note extensive cellular infiltration of alveoli and interalveolar tissue, the former consisting chiefly of the large mononuclear cells.  $\times 400$ . *B*, Section of lung of sheep 781, which was inoculated with lung material from sheep 777, naturally affected with the disease. Note similarity with figure 1, *A*, in the thickening of alveolar walls and cellular infiltration of alveoli and interalveolar tissue.  $\times 400$ .

results of exposure were apparently negative for all the experimentally exposed sheep except those just referred to, detailed information is given only for these four.

TABLE 1.—Results of tests with sheep exposed to chronic progressive pneumonia by various methods

Healthy sheep exposed (number)	Material used in exposure tests <sup>1</sup>	Method of exposure <sup>2</sup>	Incidence of pulmonary lesions found on post-mortem examination			
			Sheep with no lesions	Sheep with parasitic lesions	Sheep with slight pneumonic lesions	Sheep with lesions suggestive of chronic progressive pneumonia
			Number	Number	Number	Number
22.....	Emulsified lung tissue from naturally affected sheep.	Intrapulmonary inoculation.	13	4	2	3
11.....	do.	Intravenous inoculation.	7		3	1
3.....	do.	Intratracheal inoculation.	2	1		
9.....	Organisms from lung of naturally affected sheep.	Intrapulmonary inoculation.	5	2	2	
8.....	do.	Intravenous inoculation.	6	2		
3.....	do.	Intratracheal inoculation.	3			
9.....	do.	Feeding.	9			
2.....	Fresh blood from naturally affected sheep.	Intravenous inoculation.	2			
15.....	None	Contact.	15			
11.....	Emulsified lung tissue from experimentally exposed sheep.	Intrapulmonary inoculation.	10	1		
1.....	do.	Intravenous inoculation.	1			
1.....	Puslike material from lung of experimentally exposed sheep.	Subcutaneous inoculation.	1			
1.....	Organisms from lung of experimentally exposed sheep.	Intravenous inoculation.	1			

<sup>1</sup> In the case of the emulsified lung tissue, small portions of the mediastinal lymph gland were usually included.

<sup>2</sup> The desirability of arranging the experimentally exposed sheep in the various groups precludes showing the time elapsing between exposure and post-mortem examination of the individual animals, which varied from 3 to 12 months.

<sup>3</sup> 1 of these sheep died from acute septic infection shortly after inoculation.

The four sheep were inoculated with emulsified lung tissue, including a small amount of mediastinal lymph gland, from four of the diseased sheep. Exposure was made by the intrapulmonary method except in the case of sheep 813, which was exposed intravenously. The time elapsing from inoculation to slaughter was as follows: 8 months 4 days for sheep 781, 11 months 1 day for sheep 813, 9 months 4 days for sheep 820, and 11 months 22 days for sheep 852. None of these animals showed any definite clinical symptoms at the time of slaughter. The gross lesions found in each of the four animals were as follows:

In sheep 781, both lungs contained numerous grayish, solidified areas throughout, and, in addition, one larger area, approximately 2 inches in diameter, was observed in the postero-superior portion of the right lung.

In sheep 813, chronic pneumonic areas more or less limited in character were found in both lungs.

In sheep 820, when the thoracic cavity was opened the lungs did not collapse. Numerous adhesions were noted. The lungs contained

numerous grayish consolidated areas with more uniform solidification of the lung tissue toward the borders of the diaphragmatic lobes.

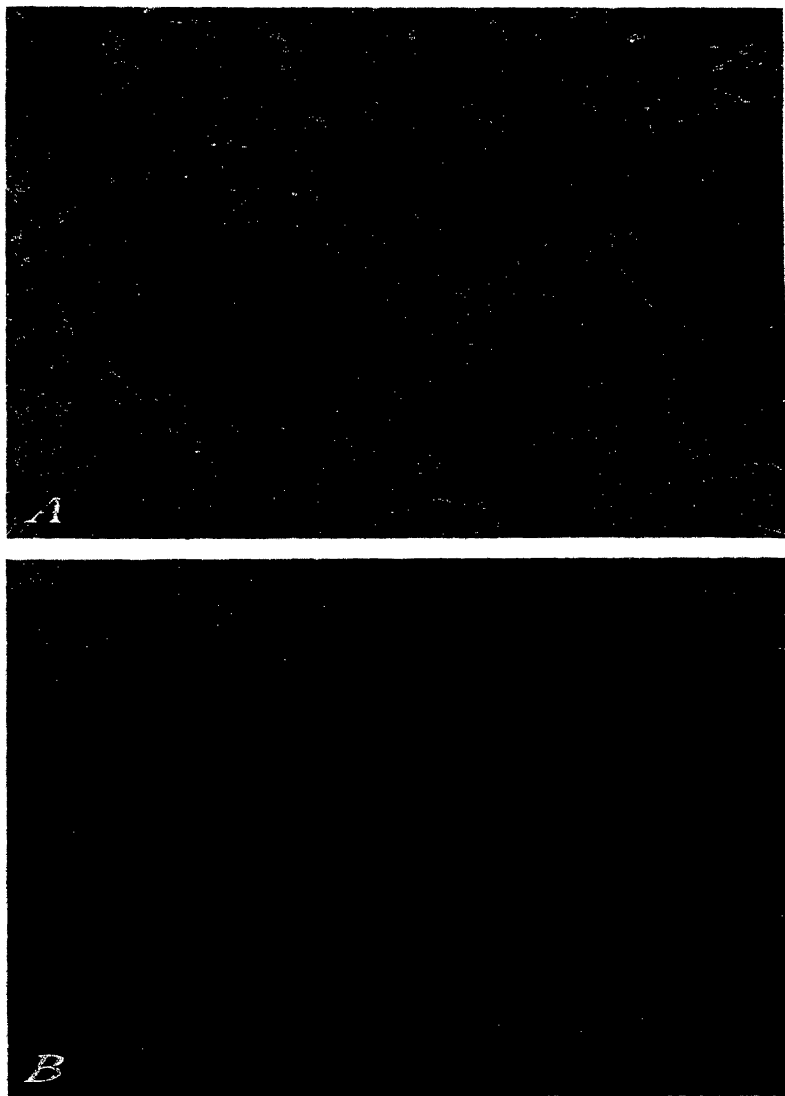


FIGURE 2.—A, Section of lung of sheep 712, naturally affected with chronic progressive pneumonia. Note typical intervalveolar lymphoid deposits.  $\times 120$ . B, Section of lung of sheep 852, which was inoculated with lung material from sheep 637, naturally affected with the disease. Note similarity with Figure 2, A, particularly with respect to the lymphoid deposits.  $\times 120$ .

On gross section the solidified areas closely simulated the lesions of chronic progressive pneumonia.

In sheep 852, when the thoracic cavity was opened the lungs did not collapse. There were some constrictions or lobulations as a

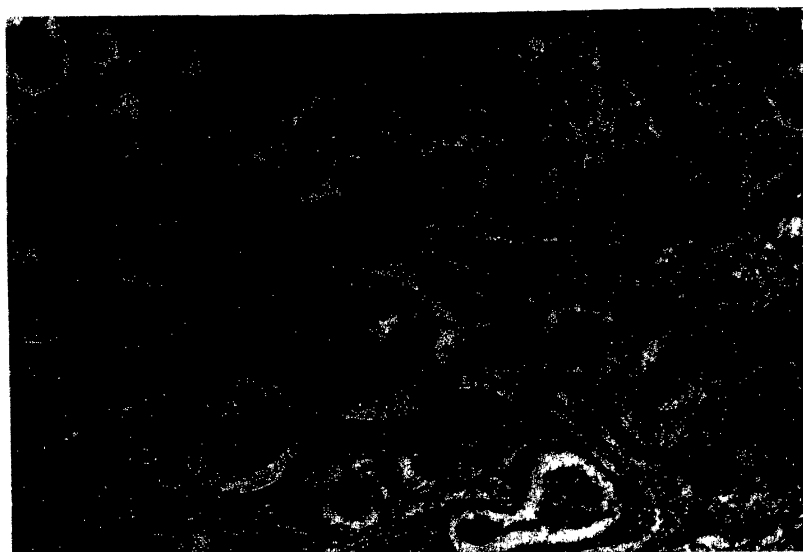


FIGURE 3.—Section of lung of sheep 728, naturally affected with chronic progressive pneumonia. Note typical bronchial proliferation.  $\times 120$ .



FIGURE 4.—Section of lung of sheep 797, naturally affected with chronic progressive pneumonia and in the later stages of the disease. Note the rather extensive fibrosis of the lung tissue  $\times 120$ .



result of fibrous proliferations. Numerous grayish solidified areas were seen throughout both lungs. Solidification was more uniform toward the superior portion and borders of the diaphragmatic lobes. The bronchial and mediastinal lymph glands appeared to be enlarged and edematous.

The bacteriological results from these four sheep were considered negative from the standpoint of pathogenic micro-organisms. Culture media inoculated from the lung tissue of one of the sheep, no. 813, remained sterile. The lung tissue of the other three yielded a limited number of organisms, none of which was found to be pathogenic.

Although there were slight variations, on the whole the pathological changes observed in the lungs of sheep 781, 813, 820, and 852, were similar. There was thickening of the alveolar walls as a result of the interalveolar cellular infiltrations. There was also cellular exudate into the alveoli, consisting mostly of the large mononuclear cells and lymphocytes. Note the similarity, in these respects, between the lung tissue of sheep 781 (fig. 1, *B*), and that of a sheep 693, naturally affected with chronic progressive pneumonia (fig. 1, *A*).

In the lung tissue of one of the sheep, no. 852, there were also rather extensive infiltrations of polymorphonuclear leucocytes. Occasional localized capillary engorgement was noted. Typical peribronchial and interalveolar lymphoid nodules were a prominent feature of the lesions in all four animals, as illustrated for sheep 852 (fig. 2, *B*). In this respect also, attention is called to the similarity between the section of lung of this sheep and that of a naturally affected sheep (fig. 2, *A*).

Epithelial proliferations of the alveoli and bronchioles were also noted, those in sheep 820 (fig. 5, *B*) being similar to those in one of the naturally affected sheep (fig. 5, *A*). These proliferations were somewhat more marked in sheep 852 (fig. 6) than in the others. In this sheep also more extensive fibrous changes were noted in the lung tissue than in any one of the other three.

Five healthy sheep approximately 1 year of age were inoculated by the intrapulmonary method with material from the lung and mediastinal lymph gland of three of the experimental sheep as follows: Two animals from sheep 852, two from sheep 820, and one from sheep 781. No inoculations were made from sheep 813. One animal died approximately 1 month after inoculation, one was held for 3 months, one for 6 months, one for 11 months, and one for 12 months. None of the five animals showed visible evidence of chronic progressive pneumonia on post-mortem examination.

#### DISCUSSION

Although a rather large number of transmission experiments were made, lesions suggestive of chronic progressive pneumonia were found in only 4 of the 96 experimentally exposed sheep. The writers believe that the lesions in these four sheep resulted from experimental exposure. Conclusive evidence of direct transmission was lacking, however, because subsequent attempts to produce similar lesions in other sheep by inoculating them with lung material from three of the four animals in question gave negative results. Similar difficulty has been experienced by other investigators of this disease. This may have been due to not having affected sheep at the proper stage

for transmission or to a lack of susceptibility in the normal sheep that were exposed to the disease. That there is a lack of susceptibility in a large percentage of sheep seems to be evidenced by the fact that

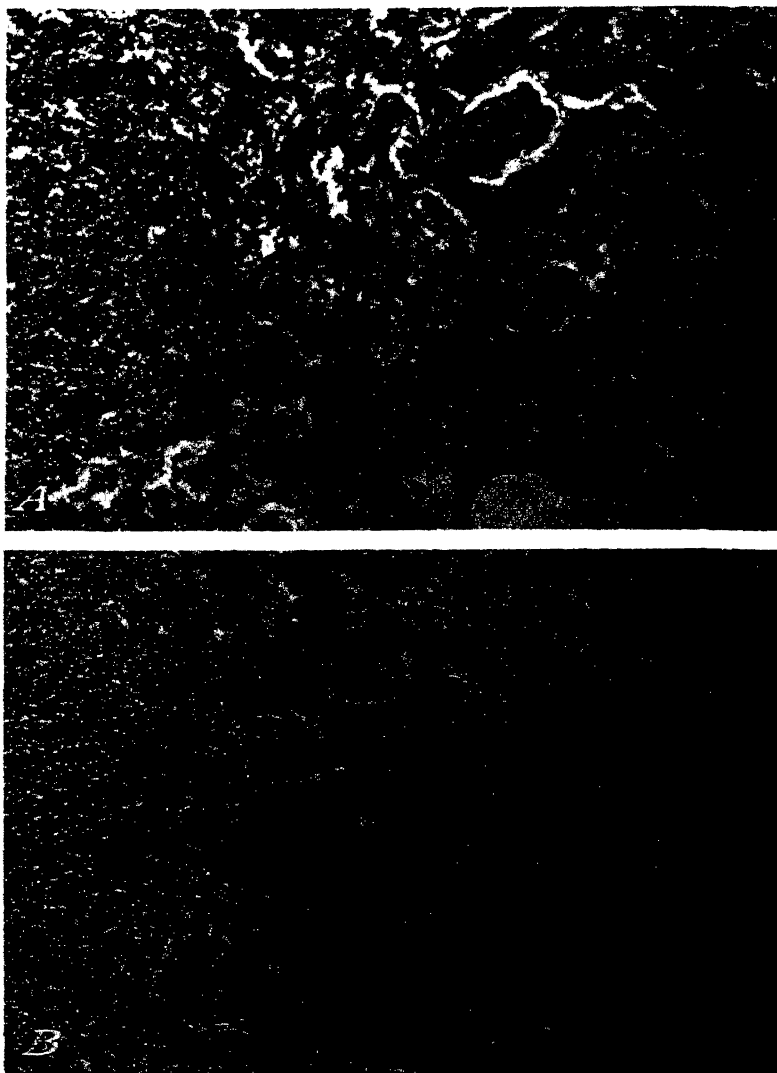


FIGURE 5.—*A*, Section of lung of sheep 829, naturally affected with chronic progressive pneumonia. Sheep 813 was inoculated with lung material from this animal.  $\times 120$ . *B*, Section of lung of sheep 820, which was inoculated with lung tissue from sheep 830, naturally affected with chronic progressive pneumonia.  $\times 120$ . Note similarity of lesions to those in lung of sheep 829 (fig. 5, *A*), particularly with regard to proliferation of bronchioles and lymphoid nodules.

only from 2 to 10 percent of the animals become affected in herds in which the disease is found. In the present experiments, the difficulty in transmission might also be accounted for by the difference

in environment at the experiment station where the transmission experiments were conducted and that of the areas where the disease occurs naturally. In the latter environment there may be predisposing influences as a result of which sheep become more susceptible, and there also seems to be a possibility that recovery may take place in some cases when the animals are removed from such environmental conditions.

Cowdry and Marsh (3) are of the opinion that there is some evidence that animals in the earliest stages of the disease may recover. In the present investigation three sheep naturally affected with the disease showed only slight evidence of it when they were slaughtered. One of these sheep was slaughtered after being held under observation



FIGURE 6.—Section of lung of sheep 852, which was inoculated with lung material from sheep 827, naturally affected with chronic progressive pneumonia. Note marked proliferation of the bronchial epithelium.  $\times 400$ .

for 9 months, and the other two after a period of 18 months. Although no well-marked symptoms were noted in these animals during the period of observation, it is presumed that some clinical evidence of the disease had been observed prior to shipment from Montana; and if so these cases strongly suggest the possibility of recovery, particularly if affected sheep are removed from their original environment during the early stages of the disease.

Records on the length of time that diseased sheep survive after being removed from their native environment show individual variation in this respect. Two of the sheep from Montana were kept at the Experiment Station at Bethesda, Md., for more than 18 months. One of these showed little clinical evidence of the disease when it was slaughtered, and on post-mortem examination the pneumonic lesions were found to be somewhat limited in extent. In the other sheep there was some evidence of difficult respiration, and somewhat more advanced lung lesions were found on post-mortem examination.



# DETERMINATION OF THE LESS REFINED MINERAL OILS ON LEAF SURFACES AFTER SPRAYING<sup>1</sup>

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## INTRODUCTION

It was shown in a previous paper (3)<sup>2</sup> that small quantities of a refined mineral oil retained by leaves after spraying could be quantitatively recovered from the leaf surfaces of camphor-trees and on pecan and Satsuma orange trees. The determinations were made with a single oil, known as Marcol. Success in the later steps of the procedure depended upon the fact that concentrated nitric acid was shown to have no appreciable action on Marcol. Treatment of the samples with this reagent was one of the important steps, and the procedure, although possessing utility for refined oils, was not claimed to be applicable to unrefined oils, since the extent of the action of the nitric acid upon this type was not demonstrated. A suitable procedure for the estimation of the less refined oils on different species of foliage has since been worked out, and is described in this paper.

## OILS STUDIED

A number of the less refined oils differing considerably from one another, and such as might be used in dormant spraying, were selected for study of the accuracy obtainable in analyses. Certain physical and chemical constants of these oils are given in table 1.

TABLE 1.—*Properties of mineral oils employed in determinations and of the highly refined Marcol used in previous experiments*

Oil	Color	Cloud test <sup>1</sup>	Pour test <sup>1</sup>	Specific gravity, 20°/20° C.	Saybolt viscosity at 37.8° C.	Volatility <sup>3</sup>	Unsulphonated residue <sup>3</sup>
		° F.	° F.		Seconds	Percent	Percent
Cayuga.....	Yellow.....	48	20	0.8841	102	1.16	62.0
Osage.....	do.....	56	35	.8898	140	.74	58.4
Red Engine.....	do.....	40	35	.8939	154	.15	55.0
L-1780.....	do.....			.8947	220	* 1.00	72.9
Alcopol.....	do.....	2	—10	.9188	221	1.74	59.6
Niantic.....	do.....	46	40	.8042	307	.25	(*)
White Rose.....	Water white.....			.8512	95	* 1.44	94.0
Marcol.....	do.....	58	30	.8419	86	1.58	95.8

<sup>1</sup> Determined according to the American Society for Testing Materials Method D89-22T (7, pp. 52-56). In the description of this method temperatures are given in degrees Fahrenheit.

<sup>2</sup> Determined according to method adopted by the Insecticide Division, but at 110° C. See Wiley (8, v. 2, p. 666).

<sup>3</sup> Determined according to method adopted by the Association of Official Agricultural Chemists (1).

<sup>4</sup> Volatility determined over 24 hours at 100° F. (37.8° C.), see Dawsey (2).

<sup>5</sup> Large amount of free carbon prevented reading the oil column after test on this oil.

The object in listing these constants is to furnish a description of the materials employed rather than to supply any data directly useful in calculating the quantity of oil on foliage. For example, the constants do not afford information concerning the oxidation, nitration, or splitting of unsaturated, aromatic, or naphthalenic compounds which may be components of the unrefined oils before treatment with nitric acid.

<sup>1</sup> Received for publication Dec. 12, 1935; issued June 1935

<sup>2</sup> Reference is made by number (italic) to Literature Cited, p. 693.

## RECOVERY OF OILS FROM CAMPHOR-TREE LEAVES

## DETERMINATION OF OPTIMUM NITRATION PERIOD

Experiments were first performed to determine the extent of the action of nitric acid upon some of the oils under the analytical conditions previously adopted. Up to the addition of nitric acid the procedure was substantially the same as that described for the recovery of refined oil from leaves of the camphor-tree (*Cinnamomum camphora*) (3). Each sample contained 100 leaf disks 30 mm in diameter, to which weighed quantities of oil had been added. After extraction the waxes were frozen out, the filtrates transferred to Babcock bottles, and the ether-alcohol solutions evaporated, so that the residues consisted only of mineral oil, natural leaf oil, and chlorophyll. The acid treatment, with which this paper is chiefly concerned, consisted in adding 6.0 cc of concentrated nitric acid to the cold oil residues in the Babcock bottles and heating the bottles in boiling water for a chosen length of time with agitation at 5-minute intervals. Since the quantity of oil present was rarely more than 100 mg, nitric acid was always present in large excess.

The volume of 6.0 cc nitric acid was chosen because of the construction of the capillary bottles. The inlet tubes on all bottles projected about two-thirds of the distance down into the lower bulbs, so that a greater volume of acid would have caused loss of the contents during the evolution of the nitrogen oxides which accompanied the oxidation. It was necessary to carry out the oxidation at high temperatures to eliminate completely the leaf materials extracted with the oil.

Table 2 shows the weights and percentages of the various unrefined petroleum oils that were undestroyed by the acid under the conditions of the experiment. In figure 1, which gives these results graphically, it may be seen that the nitric acid did not attack any two of the oils to exactly the same degree. These curves were employed in estimating the optimum heating period for each oil, so that minimum errors would be introduced into the analyses of the respective oils. For example, for Cayuga, Osage, Red Engine, Alcopol, and Niantic oils, the slopes were least after heating for 60, 60, 60, 20, and 15 minutes, respectively. In the interest of shortening the procedure, however, the following nitration periods were adopted for the respective oils: 30, 50, 30, 30, and 15 minutes.

## CHECK ANALYSES

On the basis of these estimates of the proper nitration period for each oil, a number of check analyses were carried out to establish the reliability of such methods in the determination of unknown samples. Data from series of such check analyses on four of the less refined oils are listed in table 3. Each was run a different time, and no attempt was made to maintain a consistent strength of nitric acid from day to day. It is therefore highly probable that no two oils were treated with acid of exactly the same concentration, although all the samples of each oil were treated with acid of the same strength.

TABLE 2.—*Recovery of unrefined petroleum oils after treatment with nitric acid for various periods of time in the presence of materials extracted from camphor-tree leaves*

Oil and sample no.	Nitration period	Oil added		Oil recovered	
	Minutes	Miligrams	Miligrams	Percent	
Cayuga:					
1.....	15	97.3	87.9	90.3	
2.....	30	81.4	68.8	84.5	
3.....	45	99.1	80.2	80.9	
4.....	60	109.6	85.4	77.9	
Osage					
1.....	15	93.8	81.6	87.0	
2.....	30	86.0	71.1	82.7	
3.....	45	90.9	72.3	79.5	
4.....	60	89.4	71.5	80.0	
Red Engine <sup>1</sup>					
1.....	15	96.7	95.0	98.2	
2.....	30	118.1	114.4	96.9	
3.....	45	98.9	93.9	94.9	
4.....	60	82.4	77.7	94.3	
Alcopol:					
1.....	15	88.4	72.4	81.9	
2.....	30	114.0	88.5	77.6	
3.....	45	93.1	60.5	65.0	
4.....	60	96.4	52.9	54.9	
Niantic					
1.....	15	81.5	63.6	78.0	
2.....	30	99.3	74.7	75.2	
3.....	45	80.5	63.3	78.6	
4.....	60	95.4	74.3	77.9	

<sup>1</sup> The concentration of the nitric acid used with these samples was unknown, but its specific gravity was considerably less than 1.42 because the acid was exposed to the sunlight in an open beaker for several days before use. Compare sample 2 with samples of the same oil in table 3, where stronger acid was employed

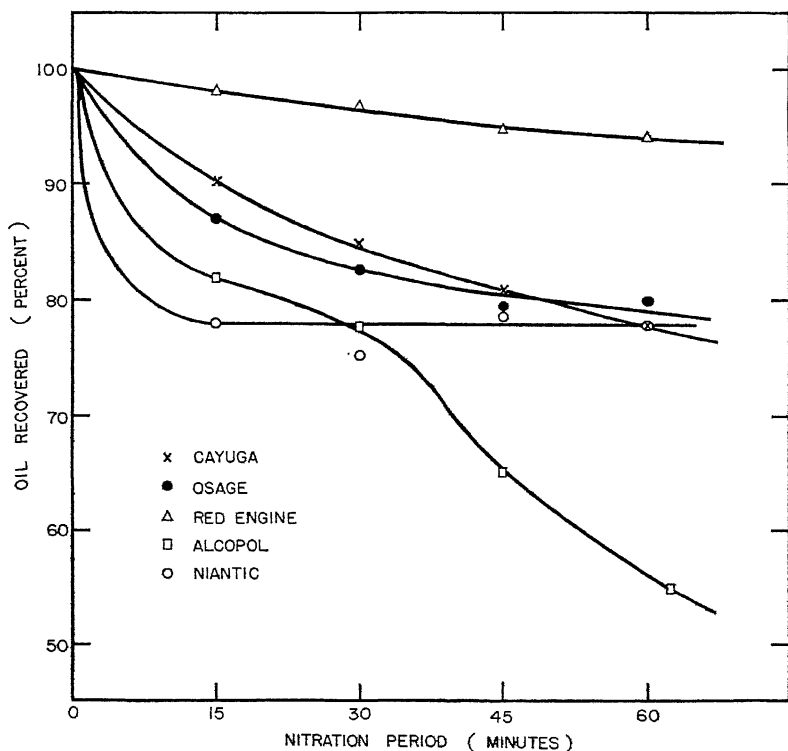


FIGURE 1.—Percentages of oils recovered after treatment of samples with nitric acid for different lengths of time. Plotted from the data in table 2.

TABLE 3—Check analyses on four unrefined mineral oils in the presence of materials extracted from camphor-tree leaves, showing the agreement between the calculated oil and that actually present

Oil and sample no	Nitration period	Oil added	Oil recovered		Calculated oil added	Error
	Minutes	Milligrams	Milligrams	Percent	Milligrams	Percent
Cayuga.....	30					
1.....		72.9	61.5	84.4	72.8	-0.1
2.....		107.2	90.6	84.5	107.2	0
3.....		81.4	68.8	84.5	81.4	0
4.....		69.6	59.0	84.8	69.6	+4
5.....		84.1	79.5	84.5	94.1	0
6.....		81.4	68.5	84.2	81.1	-4
7.....		57.9	48.8	84.3	57.8	-2
Mean.....				84.5		
Osage.....	50					
1.....		89.6	71.2	79.5	90.4	+9
2.....		89.7	69.4	77.4	88.1	-1.8
3.....		88.8	69.2	77.9	87.8	-1.1
4.....		89.4	69.9	78.2	88.7	-8
5.....		90.9	72.3	79.5	91.8	+1.0
6.....		84.8	68.3	80.5	86.7	+2.2
Mean.....				78.8		
Red Engine <sup>1</sup> .....	30					
1.....		116.6	99.0	84.9	117.0	+3
2.....		79.8	67.3	84.3	79.5	-4
3.....		98.8	83.4	84.4	98.0	-2
4.....		74.7	63.2	84.6	74.7	0
5.....		115.0	97.7	85.0	115.5	+4
6.....		104.9	88.4	84.3	104.5	-4
7.....		74.8	63.2	84.5	74.7	-1
8.....		100.3	85.0	84.7	100.5	+2
Mean.....				84.6		
Niantic.....	15					
1.....		79.4	63.8	80.4	79.9	+6
2.....		99.1	78.6	79.3	98.5	-6
3.....		95.2	76.1	79.9	95.4	+2
4.....		94.9	75.4	79.5	94.5	-4
Mean.....				79.8		

<sup>1</sup> The percentage of Red Engine oil destroyed was considerably larger than that in the similar sample 2, table 2. This is accounted for by the fact that stronger acid was employed here.

The data show that the percentages of oil undestroyed were substantially the same in different samples of the same oil. It was concluded, therefore, that it would be practical to determine unknown quantities of oil on sprayed trees simply by extracting check samples containing known quantities of oil and equal numbers of leaf disks along with the corresponding unknown samples, and, after calculating the proper correction factor, to apply it to the unknowns treated under identical conditions.

#### ALTERNATIVE METHOD

The Alcopol oil was reserved for the purpose of demonstrating an alternative method of estimating such oils in the presence of extracts of camphor-tree leaves. After the optimum nitration period of 30 minutes had been selected, several tests were run with this oil to determine what strength of acid would be required to destroy all the residual leaf materials and yet not attack the petroleum oil. A series of such tests is recorded in table 4 (samples 1 to 4). The concentration of 4 cc of nitric acid to 2 cc of water proved to be the optimum for the apparent elimination of the leaf materials, and samples 5 to 8 were run with this strength of acid to determine the reliability of this treatment. The agreement of the data in the table show that it is possible to recover completely unrefined oils on camphor-tree foliage,



provided the proper acid concentration is first determined for the specific oil and definite numbers of disks. There is a possibility, however, that the traces of undestroyed leaf oil just compensated for the petroleum oil taken up by the diluted nitric acid.

TABLE 4.—Check analyses of Alcopol oil, showing the error introduced by nitration at various concentrations of acid

Sample no. <sup>1</sup>	Nitric acid added	Water added	Oil added		Oil recovered	
	Cubic centimeters	Cubic centimeters	Milligrams	Milligrams	Percent	
1.....	6	0	114.0	88.5	77.6	
2.....	5	1	98.5	90.3	91.7	
3.....	4	2	96.1	96.0	99.9	
4.....	3	3	95.6	100.9	105.5	
5.....	4	2	94.2	94.1	99.9	
6.....	4	2	99.0	98.1	99.1	
7.....	4	2	87.5	98.2	98.4	
8.....	4	2	101.1	100.5		

<sup>1</sup> Samples 1 to 4 were trial samples. Samples 5 to 8 show the results obtained under the optimum acid concentration.

#### RECOVERY OF OILS FROM SATSUMA ORANGE LEAVES

The recovery of two of the oils was also studied in the presence of extracts of the leaf of Satsuma orange (*Citrus nobilis unshiu*), with the adoption of an optimum nitration period as in the experiments with camphor-tree leaves. In these experiments the natural waxes were frozen out from the initial extracts under the previously determined optimum conditions. The results in table 5 show that a correction factor is applicable in the determination of these less refined oils on Satsuma orange foliage. The error involved, however, although within  $\pm 4$  percent, was larger than in the case of the camphor-tree leaves, and also slightly larger than that obtained with the more highly refined Marcol when extracted with orange leaves, as reported in the earlier paper (3).

TABLE 5.—Check analyses of Red Engine and Cayuga oils in the presence of extracts of Satsuma orange leaf

Oil and sample no.	Oil added		Oil recovered		Calculated oil added	Error
	Milligrams	Milligrams	Percent	Milligrams	Percent	
Red Engine:						
1.....	112.7	79.5	70.5	111.6	-1.0	
2.....	116.1	85.4	73.6	119.9	+3.3	
3.....	78.5	55.3	70.4	77.6	-1.1	
4.....	75.5	54.6	72.3	76.7	+1.6	
5.....	93.7	66.2	70.7	92.9	-.9	
6.....	93.1	65.1	69.9	91.4	-1.8	
Mean.....			71.2			
Cayuga:						
1.....	108.5	72.9	67.2	110.7	+2.0	
2.....	89.5	58.1	64.9	88.3	-1.3	
3.....	114.8	76.7	66.8	116.5	+1.5	
4.....	62.1	39.4	63.4	50.8	-3.7	
5.....	84.7	55.3	65.3	84.0	-.8	
6.....	104.9	70.6	67.3	107.2	+2.3	
Mean.....			65.8			

#### RECOVERY OF OILS FROM CHRYSANTHEMUM LEAVES

Certain variations and improvements were introduced during the development of a suitable method for the determination of petroleum oils on chrysanthemum foliage. For these tests leaves from the Old

Rose variety of chrysanthemum (*Chrysanthemum hortorum*) plants were used. The potted plants were reared in a greenhouse and were of uniform size and age. Before extraction, samples contained 200 leaf disks 1 cm in diameter together with definite quantities of petroleum oil.

#### EFFICIENCY OF EXTRACTION

Since the oil absorbed in the chrysanthemum leaf disks was to be extracted with ethyl ether, it was necessary to ascertain the efficiency of extraction before making determinations of the petroleum oil from the plants.

In one experiment a separate set of eight plants was sprayed with a 2-percent emulsion prepared from the highly refined White Rose oil with bone glue as emulsifier. After the water from the spray had dried, four replicate samples of leaf disks were cut and allowed to stand 2 days for the oil to penetrate the leaf tissue, thus making it harder to wash the oil from the disks. They were then extracted in several ways to determine the quantities of combined oil and wax recoverable during each separate and successive washing.

The results showed that it was impractical to wash the disks more than four times because of the time consumed, but that at least 30 to 50 cc of ether should be employed each time. For example, one sample, in five successive washings with 40 cc of ether, gave 0.0942, 0.0239, 0.0056, 0.0019, and 0.0005 cc of combined oil and wax. Proportionately larger quantities of oil were taken off in the first washing with larger volumes of ether. It was also found that the longer the disks soaked the larger were the quantities of oil removed in the initial washings. The first four washings thus recovered substantially all the quickly soluble material in the samples. In all the following reported determinations the samples of chrysanthemum disks were washed four times with 35 cc of ether.

In other experiments, where approximately 24 hours elapsed between the spray application and the leaf extraction, from 90 to 95 percent of the total deposit was recovered. These tests were carried out by extracting four times with 35 cc of ether, measuring the oil so obtained, drying and grinding the partially exhausted leaf disks, washing the powdered material repeatedly for 2 hours in an extraction apparatus, and measuring and calculating the spray oil left in the leaves after the first extractions.

#### TESTS ON OMISSION OF WAX-FREEZING STEP IN PROCEDURE

Experiments were then made to ascertain whether the wax-freezing step might be omitted from the procedures employed in the studies with camphor-tree and orange leaves. It was found possible, instead of separating the wax from the petroleum oil, to measure the two together and then calculate the oil by subtraction.

A set of tests was conducted to determine the quantity of extractable wax in samples of 200 leaf disks to which known weights of White Rose petroleum oil were added. These tests consisted in filtering the ether extracts, evaporating off the solvent, transferring the residue to calibrated Babcock bottles and treating it with 5 cc of nitric acid at 100° C., and then measuring the combined oil and wax in the capillaries after filling with more nitric acid and centrifuging. It was necessary to centrifuge at about 50° because there was sufficient wax present to solidify the oil in the tubes at room temperature. The data in table 6 show that the quantity of extractable wax, in the case

of chrysanthemum leaves, is independent of the petroleum-oil content and remains constant within workable limits.

TABLE 6.—*Extractable wax in samples of chrysanthemum leaf disks to which known weights of White Rose petroleum oil were added*

Sample no.	Oil added	Oil and wax recovered	Wax, by difference
	<i>Milligrams</i>	<i>Milligrams</i>	<i>Milligrams</i>
1.....	17.5	22.0	4.5
2.....	29.8	34.2	4.4
3.....	38.0	42.2	4.2
4.....	46.6	50.6	4.0
5.....	56.1	60.8	4.7
6.....	67.0	71.9	4.9
7.....	71.3	75.8	4.5
8.....	81.3	85.4	4.1
Mean.....			4.4

Since it has been shown that the extractable wax was completely removed under the stated conditions, it follows, for samples of lesser wax content at least, that the quantity of recoverable wax would be proportional to the number of leaf disks treated.

#### CHECK ANALYSES

After the establishment of the proper conditions under which to extract chrysanthemum leaves, such that substantially complete recovery of the insecticidally active portions of mineral oil might be accomplished, and it had been shown that the usual procedure might be simplified by omitting the wax-freezing step, a series of five tests was run to determine the degree of accuracy attainable in the estimation of the oil content in check samples. One of the less refined oils, no. L-1780, was chosen for these tests. Table 7 shows the results obtained when 5 cc of nitric acid was added to each sample followed by the usual intermittent agitation and heating. After account had been taken of the corrections for the undestroyed leaf wax present because of the omission of the wax-freezing step, and for the oil destroyed by the acid treatment, the calculated quantities of oil present were found to be within  $\pm 2.5$  percent of their true values. It was also shown that the accuracy of the calculated figures was again independent of the actual oil content of the samples.

TABLE 7.—*Check analyses of oil L-1780 in the presence of chrysanthemum leaf material*

Sample no.	Oil added	Oil and wax recovered <sup>1</sup>	Oil recovered <sup>2</sup>		Calculated oil added	Error
	<i>Milligrams</i>	<i>Milligrams</i>	<i>Milligrams</i>	<i>Percent</i>	<i>Milligrams</i>	<i>Percent</i>
1.....	50.7	44.6	40.2	79.2	51.7	-2.0
2.....	65.2	64.8	60.4	77.3	64.8	-.6
3.....	77.5	63.2	58.8	75.9	75.6	-2.5
4.....	101.3	83.7	79.3	78.3	101.9	+ .6
5.....	116.8	95.8	91.4	78.3	117.5	+ .6
Mean.....				77.8		

<sup>1</sup> The specific gravity of the oil and wax mixture was assumed to be the same as that of the oil, namely, 0.8947 at 20°/20° C.

<sup>2</sup> The weight of oil recovered was obtained by subtracting from the weight of oil and wax recovered the constant 4.4 mg. obtained for the mean weight of wax recovered in the previous series of experiments with White Rose oil (table 6). It will be noted that this constant agrees well with the figure 3.8 mg. obtained by calculating the most probable values for the weight of wax and the percentage of oil recovered from the data on oil L-1780.

## DISCUSSION

There may be several reasons why greater accuracy was not obtained by the methods proposed. Possible errors in calibration of the Babcock bottles undoubtedly offer one explanation. For example, three separate determinations of volume were made for each bottle and the mean of these determinations was calculated. Thus, for one of the bottles the following figures were obtained for volume per millimeter of tube length: 0.0014159, 0.0014397, and 0.0014410 cc. The mean of these figures was 0.0014322 cc. The deviations from the mean are found to be 1.12, 0.56 and 0.63 percent, respectively. Since the error in calibration may be as large as 1.12 percent, determinations might regularly show errors of this size.

The investigations with camphor-tree and Satsuma orange leaves reported in this paper were made at New Orleans, La., while the studies with chrysanthemum leaves were carried out after the field station had been transferred to Wooster, Ohio. Since the omission of the wax-freezing step in the procedure for the chrysanthemum foliage proved to be a simplification without loss of accuracy, it must be considered to be an improvement. It would have been desirable to study the reliability of the methods for the other two species of leaves when this step was eliminated, but since no camphor-tree or orange leaves were available in the northern location such an investigation could not be undertaken. It should be possible, however, to simplify those two procedures also by omitting the freezing step, provided the wax-residue constant is first determined for given numbers of leaf disks, as illustrated in table 7.

Since the appearance of English's original method (4), followed by the improvements of Dawsey and Haas (3), two other methods for the determination of oil-spray deposit have been proposed. Swain and Green (6) have called attention to the distinction between the insecticidally active deposit (surface oil) and the total deposit (surface oil plus that absorbed in the leaf tissue). They have developed a comparatively simple method for an approximate determination of the surface oil, which involves washing leaf surfaces with methylene chloride, evaporating the solvent, and then weighing the residue consisting of plant wax and petroleum oil. According to the published results, the procedure is most applicable to determinations made immediately after the water has evaporated when there has been little time for the oil to penetrate the leaf tissue. Since petroleum oil and leaf-surface wax are known to be mutually soluble, however, uncertainty exists as to exactly what percentage of surface oil can be removed each time by the single methylene chloride washing treatment, particularly in view of the small quantities of the natural wax removed by these investigators in washing unsprayed leaves. Rohrbach (5) prefers to dry and grind citrus leaves before making extractions with petroleum ether. With the incorporation of certain steps, such as correcting for the quantity of oil destroyed in the sulphuric acid treatment in the case of sulphonatable oils, and correcting for the quantity of natural leaf substances extracted with the spray oil, his method appears to be applicable to the determination of total deposit regardless of the interval between spray application and extraction. All these methods have contributed to the solution of the problem of recovery of oil spray residue from plants.

Judging from what is known in the field at present, in routine work it would seem preferable to adopt procedures capable of giving the total spray deposit, as advocated by Rohrbach (5), rather than the surface oil, which is in itself a variable quantity dependent upon penetration of the oil into the leaf tissue as influenced by the volatility and time factors. If interest is centered upon the insecticidal action of the oil, it would also appear, from the evidence at hand, that extractions should be made as soon as possible after spraying, in order to minimize the volatilization effect, thereby obtaining definite and reproducible values for the deposit each time.

Two logical criticisms have been made of the general methods employed by English (4) and by Dawsey and Haas (3): (1) Taking large disks from the center of leaves does not give representative samples, because the oil accumulates principally along the midrib and edges of the leaves; and (2) simply washing the leaves with ethyl ether will not extract the last traces of oil from the leaf tissue. In the method for chrysanthemum leaves given in this paper the first difficulty has been largely eliminated by taking a large number of small disks over the whole leaf area so that advantage of speed in sampling has been retained. With regard to the second criticism, from 90 to 95 percent of the total oil can be recovered, at least with chrysanthemum leaves extracted soon after spraying, by the methods herein described. The procedure, therefore, offers a means for a good approximation to the total oil deposit without recourse to the laborious process of reducing foliage to a powder before extraction, and in addition does not require the use of expensive extraction apparatus.<sup>3</sup> The work is being continued at the Wooster laboratory in a study of extraction methods applicable to vegetable and animal oils as well as to petroleum oils.

#### SUMMARY

The problem of determination of the less refined petroleum oils in the presence of materials extracted from plant foliage by ethyl ether has been studied. Treatment of the leaf-extract residues containing petroleum with nitric acid under controlled conditions results in destruction of a definite proportion of the oil each time, and by using a predetermined correction factor the original quantity of the oil present can be estimated. Applications are suggested in the determination of oil deposit from the foliage of camphor-tree, Satsuma orange, and chrysanthemum plants following spraying with oil emulsions.

The method of analysis of these sulphonatable oils in the presence of chrysanthemum leaf materials has been simplified, without loss of accuracy, by omitting the usual wax-freezing step as employed in the treatment of the camphor-tree and Satsuma orange leaf extracts.

The efficiency in oil recovery in washing leaf disks taken from sprayed plants with ethyl ether has also been studied. For a limited number of washings recovery is not complete, but from 90 to 95 percent of the oil is removed even when extraction is made 24 hours after the spray application.

<sup>3</sup> Later results have shown that 100 percent of the oil deposit can be recovered by continuous washing with various solvents in an extraction apparatus, provided fresh green leaf disks are used. Drying without powdering prevents complete recovery, however, under the same conditions.

The work of other investigators is discussed in the light of the present work and the position taken that the total oil deposit is the preferable quantity to measure in the study of the insecticidal action of oils.

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# INHERITANCE OF RESISTANCE TO THE HESSIAN FLY IN THE WHEAT CROSSES DAWSON × POSO AND DAW- SON × BIG CLUB<sup>1</sup>

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## INTRODUCTION

A promising and economical means of controlling the injury to commercial California wheats caused by the hessian fly, *Phytophaga destructor* (Say), lies in breeding varieties resistant to its attack. Such an objective is achieved most easily when the plant breeder uses resistant varieties in which resistance is due to the smallest possible number of factors. The existence of fly-resistant varieties among the common wheats has been demonstrated by many workers. That such resistance is due to a definite number of genetic factors has not yet been shown conclusively. In this preliminary paper data from two crosses are presented which the writers believe indicate the existence of two such genetic factors.

This breeding program was started to develop fly-resistant club wheats for the Montezuma Hills district of Solano County, Calif. It is desirable to grow a club type of wheat in this district, because such wheat does not suffer from shattering from the high winds which prevail there. The two club wheats, Big Club and Poso, which are now grown there are susceptible to the hessian fly. The aim of the breeding program is therefore to add the character of fly resistance to these two varieties and at the same time retain the desirable qualities that they now possess. To attain this end backcrosses will be made to the commercial type and the hybrids thus obtained subjected to rigorous selection under heavy fly infestation.

## REVIEW OF LITERATURE

Not a single instance has been found in the literature where the inheritance of resistance to the hessian fly has been definitely put on a factorial basis. The following two statements, however, have a bearing on the problem. Painter, Salmon, and Parker<sup>2</sup> state:

The most important fact brought out by these data is that resistance is an inherited character which may be combined with other desirable ones and that fly resistance is not closely linked with any observed agronomic character such as awn type and kernel texture.

Later Parker and Painter<sup>3</sup> stated that—

Studies of crosses between resistant and susceptible wheat varieties have shown that fly resistance is a heritable character, probably governed by multiple factors \* \* \*.

<sup>1</sup> Received for publication Jan. 31, 1936; issued June 1936.

<sup>2</sup> PAINTER, R. H., SALMON, S. O., and PARKER, J. H. RESISTANCE OF VARIETIES OF WINTER WHEAT TO HESSIAN FLY, *PHYTOPHAGA DESTRUCTOR* (SAY). Kans. Agr. Expt. Sta. Tech. Bull. 27, 68 pp., illus. 1931. See p. 39.

<sup>3</sup> PARKER, J. H., and PAINTER, R. H. INSECT RESISTANCE IN CROP PLANTS. Sixth Internat. Cong. Genetics, Ithaca, N. Y., Proc., v. 2, pp. 160-162. 1932.

## MATERIAL AND METHODS

The parents used in the crosses under discussion are the susceptible varieties Poso (C. I. no. 8891)<sup>4</sup> and Big Club (C. I. no. 11761) and the resistant variety Dawson (C. I. no. 3342). Pure lines of all parents were used. Poso and Big Club are white-kerneled club varieties having the spring habit type of growth and are well adapted to the Montezuma Hills district. Dawson is a white-kerneled, lax-headed variety having the winter habit type of growth.

The original crosses were made in 1931 at Davis, Calif. The  $F_1$  and  $F_2$  generations were grown at the same place in 1932 and 1933. For the purpose of genetic interpretation the  $F_2$  plants were classified on the basis of their behavior in  $F_3$  rows. The  $F_3$  rows, averaging 75 plants per row, were grown at Birds Landing, in the fly-infested Montezuma Hills district. The seed was sown in December 1933, and the plants were harvested in May and June 1934. The plants therefore made their growth during the winter and early spring and were in receptive condition at the time of infestation, which is normally heavy in that locality. The susceptible and resistant parents were included alternately every eleventh row as checks among the  $F_3$  rows for each cross. A population of  $F_2$  plants of each cross was also grown in 1934.

In the vicinity of Birds Landing the hessian fly usually makes its major flight between the last of February and the early part of April, while the plants are developing from the rosette through the jointing stage. A second flight, from a supplementary brood of flies, generally occurs late in April, or early in May in wet years. Since the natural infestation is usually very high in this district, it has not been necessary to supplement the natural infestation with an artificial one.

The plant was used as the unit in making genetic interpretations. The plants were classified as infested or noninfested depending on whether or not puparia were present. A plant bearing no puparia was considered as resistant.

It will be shown later that a small percentage of plants of the susceptible parents were not infested. These are believed to have escaped infestation, and are not considered as resistant. Packard<sup>5</sup> showed that when progeny of such escaped plants were tested the following year, they did not possess any decidedly resistant qualities. Although he obtained one plant selection which showed a consistently lower percentage of infestation, there are good reasons for believing that this selection was not of the original parent variety but was either a field hybrid or an admixture.

## EXPERIMENTAL RESULTS

## DETERMINATION OF FACTORIAL BASIS FOR INHERITANCE OF RESISTANCE

The percentages of plants of the parent varieties having hessian fly puparia for the period 1932-34 are given in table 1. The Dawson parent shows a negligible infestation as compared with the other two varieties. The Dawson parent has been tested for a longer period than table 1 indicates, and has always shown a very low infestation. Likewise Big Club has always shown a high infestation over a longer period. The data for the Poso parent do not extend beyond the 3-year period.

<sup>4</sup> Accession number of the Division of Cereal Crops and Diseases, Bureau of Plant Industry.

<sup>5</sup> PACKARD, C. M. THE HESSIAN FLY IN CALIFORNIA. U. S. Dept. Agr. Tech. Bull. 81, 26 pp., illus. 1928.



TABLE 1.—*Infestation of hessian fly puparia in Dawson, Poso, and Big Club wheats, when grown in the field at Birds Landing, Calif., for the years 1932-34*

Variety	1932		1933		1934		Average plants with puparia, 1932-34 <sup>1</sup>
	Plants examined	Plants with puparia	Plants examined	Plants with puparia	Plants examined	Plants with puparia	
Dawson.....	Number 100	Percent 1.0	Number 50	Percent 0.0	Number 760	Percent 0.4	Percent 0.4
Poso.....	100	90.0	89	84.3	869	96.9	95.2
Big Club.....	100	92.0	195	91.8	500	93.4	96.6

<sup>1</sup> Weighted averages.

The F<sub>1</sub> plants from the two crosses were not tested for fly reaction. An F<sub>2</sub> population of 160 plants of Dawson × Poso and 125 plants of Dawson × Big Club was grown subject to fly infestation in 1934. The data for both the present varieties and the crosses are shown in table 2.

TABLE 2.—*Fly-infested plants in the parents and the F<sub>2</sub> generation of the crosses Dawson × Poso and Dawson × Big Club, when grown in the field at Birds Landing, Calif., in 1934*

Parent or cross	Total plants	Fly-infested plants	
	Number	Number	Percent
Dawson.....	760	3	0.4
Poso.....	869	842	96.9
Big Club.....	500	492	98.4
Dawson × Poso.....	160	22	13.7
Dawson × Big Club.....	125	23	18.4

As indicated previously, the genetic classification of the F<sub>2</sub> plants is based on their behavior in F<sub>3</sub> rows. There were 185 F<sub>3</sub> rows grown of each cross. The fly infestation was uniform throughout the nursery, as indicated by the parent rows which were included alternately every eleventh row as checks. The percentages of fly infestation for the alternate parent checks were as follows: In the Dawson × Poso nursery, 99, 1, 97, 0, 100, 0, 94, 0, 95, 0, 97, 0, 99, 0, 94, 1, 97, 1, 98; and in the Dawson × Big Club nursery, 100, 0, 100, 0, 96, 0, 98, 0, 98, 0, 98, 0, 96, 0, 98, 0, 100, 0, 100. The low percentages are for Dawson. The data for the infestation of the F<sub>3</sub> rows of both crosses are summarized in 5-percent classes in table 3, and are shown graphically in figure 1.

On the assumption of an arbitrary minimum at 77.5 percent infestation, this point was used in dividing the rows into two groups, according to whether more or less than 77.5 percent of the plants were infested. Since only 10 rows were grown of each susceptible parent, the range distribution for such rows is not well established. Therefore, if only those hybrid rows are considered susceptible which fall within the range of the susceptible parent, some errors of classification might result. Again, the range of susceptibility of the rows of susceptible parents is based on the relation of the two postulated

susceptible genes to a constant reaction system of other genes. Since this is not the case in rows of hybrids, the range may be extended so that a minimum somewhat beyond the range of the susceptible parent may be justified.

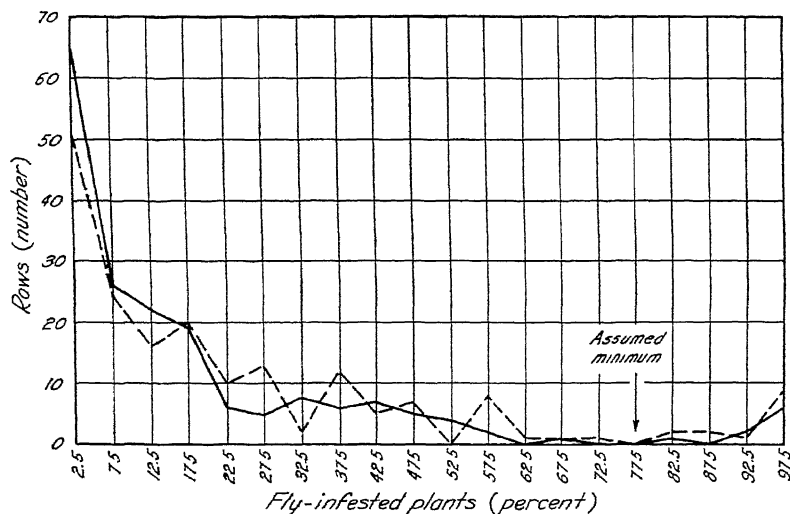


FIGURE 1.—Distribution of  $F_1$  rows of the crosses Dawson  $\times$  Poso (solid line) and Dawson  $\times$  Big Club (broken line) in 5-percent classes of infestation with hessian-fly puparia.

TABLE 3.—Distribution of the parent and  $F_1$  rows of the wheat crosses Dawson  $\times$  Poso and Dawson  $\times$  Big Club in 5-percent classes of hessian-fly infestation, when grown in the field at Birds Landing, Calif., in 1934

Parent or cross	Rows in indicated classes of fly infestation <sup>1</sup>										
	2 5 percent		7. 5 percent	12 5 percent	17. 5 percent	22 5 percent	27. 5 percent	32. 5 percent	37. 5 percent	42. 5 percent	47. 5 percent
	0 percent	0+ to 5 percent									
Dawson.....	No.	No.	No.	No	No	No	No.	No.	No	No.	No.
Poso.....	15	3									
Big Club.....											
Dawson X Poso.....	19	46	26	22	19	6	5	8	6	7	5
Dawson X Big Club.....	18	33	24	16	20	10	13	2	12	5	7

Parent or cross	Rows in indicated classes of fly infestation <sup>1</sup>										Total rows
	52. 5 percent	57 5 percent	62 5 percent	67. 5 percent	72. 5 percent	77. 5 percent	82 5 percent	87 5 percent	92. 5 percent	97 5 percent	
Dawson.....	No.	No.	No.	No.	No.	No.	No.	No.	No.	No.	No.
Poso.....									2	8	18
Big Club.....										10	10
Dawson X Poso.....	4	2	0	1	0	0	1	0	2	6	135
Dawson X Big Club.....	0	8	1	1	1	0	2	2	1	9	185

<sup>1</sup> Percentages given are class centers.

The 185  $F_2$  rows (or  $F_2$  plants) of each of the two crosses then group themselves into the ratios 176:9 and 171:14 for the Dawson  $\times$  Poso and Dawson  $\times$  Big Club crosses, respectively. On the basis of a two-factor 15:1 theoretical genetic ratio, the numbers to be expected are 173.4:11.6. The actual and theoretical ratios for both crosses agree within the limits of the experimental error. Under the conditions of these experiments the resistance of Dawson is therefore interpreted as being controlled by two genetic factors.

The interpretation of the data applies only to the conditions under which the experiments were made, and no prediction can be made as to the behavior of the parent or hybrid material when grown under other conditions. The data furnish no idea as to the fundamental nature of resistance. The answer to that problem might be of some help in the breeding program, but it is not necessary for such a program.

#### ONTOGENY OF THE FLY ON SUSCEPTIBLE AND RESISTANT PLANTS

The normal ontogeny of the hessian fly on susceptible plants may be briefly described as follows: The adult flies emerge from the puparia and mate during February and March, and the females lay their eggs on the leaves of the growing crop. In a few days the red eggs hatch, and the salmon-colored young larvae crawl down between the culm and leaf sheath and begin to feed. After feeding for 2 to 3 weeks, the larvae, then glistening white, form puparia and enter a quiescent stage, or what is commonly called a "flaxseed." During the long, dry summer and fall, these "flaxseeds" remain unchanged in the same stubble and straw that the young larvae had occupied while sucking their food from the growing stems. Late in the winter or early in the spring of the following year the larvae pupate, and in 2 or 3 weeks the adults emerge to repeat the life cycle.

On resistant plants the life cycle is not completed. The life of the insect appears to be normal until the newly hatched red larvae reach the feeding position between the leaf sheath and culm. Shortly after this the larvae die. It is this fact that points to the possibility of producing commercial wheats that will be free from fly attack.

#### SUMMARY

An attempt is being made in California to breed varieties of wheat that will possess resistance to infestation by the hessian fly. The existence of resistant varieties among common wheats has been demonstrated by many workers, but the number of genetic factors involved in such resistance has not previously been conclusively shown.

Selections from the variety Dawson are shown to be highly resistant to fly attack, and the varieties Poso and Big Club are very susceptible under California conditions. The crosses Dawson  $\times$  Poso and Dawson  $\times$  Big Club show inheritance of resistance in the  $F_2$  generation when classified on their behavior in  $F_2$  rows, in a ratio closely approximating the theoretical 15:1 ratio occurring when two factors are involved.

The ontogeny of the hessian fly in relation to both resistant and susceptible varieties is discussed briefly.

Under the conditions of the experiments as described in the text, the data for the two crosses studied indicate that resistance to the hessian fly in the Dawson variety is heritable and that it is controlled by two genetic factors.



# QUANTITATIVE DEMONSTRATION OF THE PRESENCE OF SPORES OF *BACILLUS LARVAE* IN HONEY CONTAMINATED BY CONTACT WITH AMERICAN FOUL-BROOD<sup>1</sup>

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## INTRODUCTION

In a previous paper<sup>3</sup> the writer showed that it is possible to demonstrate the presence of spores of *Bacillus larvae*, the cause of American foulbrood, in samples of commercial honey that have had contact with American foulbrood in the course of their production or preparation for the market. Since this work was reported, 25 additional samples, making a total of 212 samples of commercial honey, obtained on the open market from 28 States and 2 Territories have been examined by the same method, and spores of *B. larvae* have been found in 17, or 8 percent, of these samples.<sup>4</sup> In most cases the spores were present in relatively small numbers.

The method of examination used in the work thus far reported gave only a qualitative indication of the number of spores present, the observations being recorded as showing "the presence of a sufficient number of spores resembling spores of *B. larvae* to be designated as positive."<sup>5</sup> This amounted to from one or two definite spores to a very few spores seen in numerous microscopic fields of each stained sediment examined. The primary object was to demonstrate only their presence or absence. It was assumed that in most cases the number of spores found was considerably smaller than would be found in honey containing numbers comparable with the observed minimum infective dose of 50,000,000 per liter.

The only way of demonstrating the accuracy of this assumption has been to feed such "positive" samples of commercial honey to healthy colonies of bees. This was done with 15 of the 16 samples in which spores were demonstrated, and only 1 sample, or 6.7 percent, was found to contain sufficient infection to produce the disease in a healthy colony. These investigations indicate that the requirement of certification of honey, as has been proposed and even placed in operation in certain States, is not a justifiable measure in the control of American foulbrood under the present conditions of inspection and control of disease in this country.

To permit a more accurate, quantitative study of the infectivity of honey that has been in contact with American foulbrood, on the

<sup>1</sup> Received for publication Jan. 27, 1936; issued June 1936. This investigation was carried on at the Intermountain States laboratory of the Division of Bee Culture, which is maintained cooperatively by the University of Wyoming and the Bureau of Entomology and Plant Quarantine, U. S. Department of Agriculture.

<sup>2</sup> Acknowledgments are due to F. R. Hall, associate professor of commerce, University of Wyoming, for advice and assistance in the statistical analysis of the data.

<sup>3</sup> STURTEVANT, A. P. RELATION OF COMMERCIAL HONEY TO THE SPREAD OF AMERICAN FOULBROOD. Jour. Agr. Research 45: 257-265, illus. 1932.

<sup>4</sup> STURTEVANT, A. P. HONEY OF THE INTERMOUNTAIN REGION. Gleanings Bee Cult. 63: 463-468, illus. 1935.

<sup>5</sup> STURTEVANT, A. P. See footnote 3.

basis of its spore content—that is, a detailed study of the distribution of spores of *B. larvae* in the honey from infected hives or apiaries, or in commercial honey obtained on the open market, or of the effect of mixing infected honey with disease-free honey in the course of production or blending and preparation for the market—a more detailed investigation has been made of the spore content of honey containing approximately known numbers of spores. This has been accomplished by an improved and more accurate method of determining the number of spores in such honey, and the accuracy of the results and method has been demonstrated by means of a statistical analysis of the data obtained.

## METHOD OF OBTAINING THE DATA

### PREPARATION OF SAMPLES OF HONEY

A series of samples of honey containing approximately known numbers of spores per cubic centimeter were prepared in the manner described previously,<sup>6</sup> by adding to 100-cc quantities of spore-free honey the necessary quantities of various dilutions of a stock suspension of spores of *Bacillus larvae* containing approximately 5,000,000,000 spores per cubic centimeter. Five samples of honey were prepared in this way containing approximately 1,000,000, 800,000, 500,000, 300,000, and 50,000 spores per cubic centimeter, respectively. These samples, each considered as a unit and not as a dilution of the 1,000,000-spore sample, were heated in a water bath to 120°–130° F., and then thoroughly mixed with a mechanical stirrer for 5 minutes. Duplicate 5-cc quantities of each sample were then placed in 50-cc conical centrifuge tubes, and 45 cc of distilled water of approximately the same temperature was added. When the honey and water were completely mixed, the samples were centrifuged at 2,000 revolutions per minute for 45 minutes. All but about 1 cc of the supernatant honey-water solution of each sample was then removed by means of a pipette and suction. Again approximately 45 cc of distilled water was added, and after thorough mixing the suspensions were centrifuged for 30 minutes longer. The removal of the supernatant solution was repeated until all but approximately 0.1 cc<sup>7</sup> of the water had been removed from each centrifuge tube, and each sample of sediment was completely suspended in this remaining quantity of water by blowing gently through a capillary pipette dipped into the water. Duplicate 0.01-cc quantities of each suspension were then transferred with the capillary pipette (calibrated to deliver 0.01 cc) to microscope cover glasses. Circular cover glasses, size 12, no. 1 thickness, having an area of 1.13 cm<sup>2</sup>, proved satisfactory for this purpose. A small (2 to 3 mm) loopful of carbolfuchsin stain was added to the drop of suspension on the cover glass and thoroughly mixed with it. This stained liquid was then spread uniformly over a 1-cm<sup>2</sup> area of the cover glass, a narrow ring at the outside edge being left uncovered. The smears were allowed to dry in the air and were then mounted on microscope slides either with water or, preferably, with Canada balsam, for examination under the microscope. These stained smears were not washed in water, as this might have caused some spores to be lost.

<sup>6</sup> STURTEVANT, A. P. See footnote 3.

<sup>7</sup> A mark was placed on the outside of the conical centrifuge tubes to indicate the 0.1-cc volume.

The foregoing process gives a concentration of spores in the sediment from the 5-cc samples of honey suspended in 0.1 cc of water, or one-fiftieth the original volume.

## METHOD OF COUNTING SPORES

A method similar to that of Breed and Brew<sup>8</sup> for counting bacteria in milk was used for counting the spores of *Bacillus larvæ* in these stained smears. This method is similar to that described in a previous paper<sup>9</sup> and is represented by the formula

$$\text{Number of spores per cubic centimeter} = \frac{KNX \times 100 \times D}{N}$$

where  $K$  is the factor for the number of circular fields per 1-cm<sup>2</sup> area,  $N$  is the number of circular fields counted,  $X$  is the actual mean number of spores per field, 100 is the factor that gives the number of spores per cubic centimeter from 0.01 cc of the suspension, and  $D$  is the dilution.

TABLE 1.—Spore counts in stained smears of the sediments resulting from the centrifuging of duplicate 5-cc portions of five samples of honey containing known numbers of spores of *Bacillus larvæ*

Field no.	Spore counts in samples <sup>1</sup> containing the indicated number of spores per cubic centimeter									
	50,000		300,000		500,000		800,000		1,000,000	
	A	B	A	B	A	B	A	B	A	B
1.....	2	1	7	8	14	15	19	21	24	21
2.....	2	2	8	9	12	12	18	20	24	29
3.....	1	1	8	10	12	13	24	18	26	38
4.....	0	2	7	5	10	15	22	18	23	24
5.....	2	0	9	6	10	11	20	20	30	34
6.....	1	3	9	7	15	13	18	17	23	29
7.....	0	1	8	7	15	14	21	18	26	24
8.....	2	1	7	8	12	13	27	17	29	21
9.....	1	0	8	6	16	16	19	21	19	26
10.....	0	2	9	12	17	13	21	20	30	31
11.....	1	1	7	11	14	12	16	19	25	26
12.....	1	3	10	6	16	12	21	20	28	26
13.....	1	3	9	5	13	10	22	22	27	33
14.....	2	1	7	9	18	14	25	24	25	22
15.....	1	0	8	10	12	13	21	25	29	26
16.....	2	1	8	5	10	11	20	25	24	26
17.....	1	0	6	9	11	16	18	28	35	23
18.....	2	0	5	6	13	14	26	23	24	28
19.....	0	2	7	10	17	16	24	23	25	34
20.....	0	1	9	5	13	18	26	28	27	30
21.....	2	1	10	15	13	15	16	23	25	32
22.....	2	3	11	10	8	16	19	18	26	25
23.....	1	1	7	8	18	10	20	16	27	25
24.....	3	1	6	5	15	12	18	22	28	34
25.....	1	1	7	9	12	15	21	21	29	28
26.....	0	1	6	7	15	10	21	20	27	22
27.....	1	1	5	10	10	14	26	18	34	28
28.....	2	1	7	8	12	15	22	26	22	27
29.....	2	2	8	10	11	11	22	28	29	28
30.....	2	2	10	8	12	11	25	22	21	30
Total.....	38	39	233	244	393	400	638	641	791	835
Total for 60 fields.....	77		477		793		1,279		1,623	
Mean number of spores per field.....	1.2833		7.9500		13.2167		21.3167		27.1000	

<sup>1</sup> A and B represent duplicate portions of the samples.

<sup>8</sup> BREED, R. S., and BREW, J. D. COUNTING BACTERIA BY MEANS OF THE MICROSCOPE. N. Y. State Agr. Expt. Sta. Tech. Bull. 49, 31 pp., illus. 1916.

<sup>9</sup> STURTEVANT, A. P. See footnote 3.

An ocular micrometer disk, such as is used for counting bacteria in milk, was used in counting spores in the fields of the stained smears. The area of the circle etched on this disk was found to be 0.00006082 cm<sup>2</sup> when used in a binocular microscope with 15 × paired eyepieces and a 1.8-mm oil-immersion objective. Therefore, the factor *K* became 16,441.96.

The spores in 30 fields from each of the duplicate smears were counted, making a total of 60 fields (*N*) for each honey-spore sample. The fields were counted at random from various parts of the smear. From these counts the actual mean number of spores per field recovered in 60 fields for each honey-spore sample was determined (table 1).

Substituting the values for *K* and *N* and 0.02 (1/50) for *D*, the spore dilution in the foregoing formula gives

Number of spores per cubic centimeter

$$= \frac{16,442 \times 60X \times 100 \times 0.02}{60} = 32,884X$$

#### COMPUTATION OF THEORETICAL MEAN NUMBER OF SPORES PER FIELD

The theoretical mean numbers of spores per field that should be recovered from each of five honey-spore samples used, under ideal conditions where there is no loss of spores during the process, were calculated by the foregoing formula, which for this purpose may be stated as follows:

$$X = \frac{\text{Number of spores per cubic centimeter}}{32,884}$$

*X* now designates the theoretical mean number of spores per field. In table 2 these values are given in comparison with the corresponding actual mean number of spores per field for each honey-spore sample.

TABLE 2.—Relation between the actual and the theoretical mean numbers of spores of *Bacillus larvae* per field recovered from five samples of honey containing known numbers of spores per cubic centimeter

Spores per cubic centimeter in sample (number)	Mean spores per field			Ratio of actual mean to theoretical mean
	Theoretical	Actual	Standard deviation	
	Number	Number	Number	Percent
1,000,000.....	30.4100	27.1000±0.3554	4.0812	89.12
500,000.....	24.3279	21.3167±.2751	3.1596	87.62
500,000.....	15.2050	13.2167±.2011	2.3100	86.92
500,000.....	9.1230	7.9500±.1708	1.9615	87.14
50,000.....	1.5205	1.2833±.0747	.8582	84.40

#### RESULTS OBTAINED BY USE OF THE METHOD

By the method used, the actual mean number of spores per field obtained by counting 60 fields from each honey-spore sample differed from the calculated theoretical mean number of spores per field by 10.88 percent for the honey containing 1,000,000 spores per cubic centimeter to 15.60 percent for the honey containing 50,000 spores per



cubic centimeter (table 2). This difference, which is relatively constant for each sample, may be due to the fact that some spores are lost during the centrifuging, but more probably to the fact that a certain proportion of the spores in each smear are covered up and not seen in the masses of stained debris always present even in honey of the highest quality.

## DETERMINATION OF ACCURACY OF THE METHOD

### STATISTICAL ANALYSIS OF THE DATA

Since the data obtained for the actual mean number of spores per field (table 1) for each honey-spore sample, if plotted against the data calculated for the theoretical mean number of spores per field (table 2), give practically a straight line having a trend similar to that of a line plotted for the theoretical data alone, the relation between the theoretical means and the actual means, for the five honey-spore samples used, was determined by the customary statistical methods.

The standard deviation and the probable error for the actual mean number of spores per field were determined from frequency tables prepared from the original data (table 1) for each honey-spore sample used<sup>10</sup> (table 2). The actual means were derived from large samples (60 fields each), and the calculated probable errors and standard deviations were shown statistically to be small.

The coefficient of correlation<sup>11</sup> between the values for the actual mean number and those for the theoretical mean number of spores per field for each sample as given in table 2 was found to be  $0.9999 \pm 0.0001$ .

The relation between the actual mean number of spores per field recovered from each honey-spore sample and the corresponding most probable values estimated from the theoretical mean number of spores per field for each sample was determined by use of the regression equation for the actual mean number of spores. This was found to be  $\bar{Y} = 0.8905X - 0.1791$ . Substituting the various values of the theoretical mean number of spores per field (table 2) for  $X$  in this equation gave the most probable estimated values for the actual mean number of spores per field ( $\bar{Y}$ ) that should have been recovered from each sample (table 3). These most probable estimated values were found to be in excellent agreement with the actual values obtained.

TABLE 3.—*Theoretical and actual mean numbers of spores per field and the most probable estimated theoretical and actual mean numbers of spores per field*

Number of spores per cubic centimeter in sample	Mean number of spores per field			
	Theoretical	Estimated theoretical	Actual	Estimated actual
1,000,000.....	30.4100	30.6313	27.1000	26.9010
800,000.....	24.3280	24.1378	21.3167	21.4859
500,000.....	15.2050	15.0431	15.2167	13.3610
300,000.....	9.1230	9.1297	7.9800	7.9440
50,000.....	1.5205	1.6443	1.2382	1.1740

<sup>10</sup> CHADDOCK, R. E. *PRINCIPLES AND METHODS OF STATISTICS*. pp. 160-164, 240-241. Boston, New York [etc.]. 1925.

<sup>11</sup> CROXTON, F. E., and COWDEN, D. J. *PRACTICAL BUSINESS STATISTICS*. p. 416. New York. 1934.

The purpose of this investigation, however, was to develop an equation with which, if the actual mean number of spores per field is obtained with sufficient accuracy, the theoretical number of spores per field may be estimated, thereby giving the data necessary for estimating the number of spores per cubic centimeter in an unknown sample of honey. The regression equation or the theoretical mean number of spores per field can be used for this purpose, and was found to be  $\bar{X}=1.1228Y+0.2034$ . Substituting for  $Y$  in this equation, the various values of the actual mean number of spores per field, as obtained in table 1, gave the most probable estimated values for the theoretical mean number of spores per field that should be obtained from the actual counts for each honey-spore sample (table 3). By this method of estimation these values were found to agree closely with the original calculated values for the theoretical mean number of spores per field for each honey-spore sample (table 2).

#### DETERMINATION OF PERMISSIBLE LIMITS OF ERROR

The analysis of the data so far indicates the accuracy of the method outlined above for determining the most probable actual mean spore count per field from the mean of 60 fields counted. Variations in the counts may occur in individual samples, however, owing to the failure to recover all the spores, as stated previously.

The permissible limits of error in the statistical analysis of such cases are customarily determined by use of the standard error of estimate. This, for the most probable estimated actual means derived from the theoretical means, was found to be small,  $\pm 0.1298$  spore, and indicates the closeness with which new estimated values may be expected to approximate the true but unknown values. Since two of the five actual means fall within  $\pm 0.1298$  spore of the estimated actual means while the other three are only from 0.11 to 0.26 percent outside this zone, within which approximately two-thirds of the observations may be expected to fall in relation to the most probable values, a sufficient accuracy for the method is indicated.

The standard error of estimate for the most probable theoretical means derived from the actual means (which were found to agree closely with the estimated actual means) was found to be  $\pm 0.1458$  spore. As is to be expected in this case, again two of the original theoretical means fall within the zone of  $\pm 0.1458$  spore while the other three are only from 0.11 to 0.25 percent outside this zone. However, since  $\pm 3$  times the standard error of estimate, which should include 99.7 percent of all observations, is used customarily in delineating the largest error to which statistical analyses of this type are subject, it is found that all the theoretical means fall well within this zone, or within  $\pm 0.4374$  spore. This indicates the probable accuracy of estimating the number of spores per cubic centimeter in an unknown sample by calculating the most probable theoretical number of spores per field from the actual mean number counted.

#### PRACTICAL APPLICATION OF THE METHOD

In a previous paper <sup>12</sup> it was shown that during observations covering 5 years no cases of American foulbrood developed in 19 colonies of bees fed less than approximately 50,000,000 spores of *Bacillus*

<sup>12</sup> STURTEVANT, A. P. See table 1 of reference in footnote 3.

larvae in 1 liter of sugar sirup, or less than 50,000 spores per cubic centimeter. Of 11 colonies fed 50,000 spores per cubic centimeter, 2 developed disease and 9 remained healthy; of 6 colonies fed 75,000 per cubic centimeter, 3 developed positive disease and 1 probable disease, and 2 remained healthy; of 6 colonies fed 100,000 per cubic centimeter, 2 were positive, 1 probable, and 3 remained healthy; of 4 colonies fed 200,000 spores per cubic centimeter, 3 were positive and 1 probable. Thus it was assumed that 50,000 spores per cubic centimeter of sirup could be considered the critical number or minimum infectious dose of spores that will produce disease, when 1 liter is used as the unit volume to be fed.

Since the foregoing analysis of the data indicates, by the method of estimating used, that the actual mean number of spores per field falls well within the limits of permissible error for the estimated actual means ( $\pm 3$  times the standard error of estimate), the most probable value for such a mean for use in determining the number of spores per cubic centimeter of an unknown sample is the actual mean number of spores per field determined by counting 30 fields each from stained smears from two centrifuged sediments of this sample. If the formula  $\bar{X} = 1.1228Y + 0.2034$  is used to estimate  $\bar{X}$ , the most probable theoretical number of spores that should have been recovered, when  $Y$  represents the actual mean number of spores per field, and if this value is then multiplied by 32,884, the most probable number of spores per cubic centimeter in the unknown sample can be calculated. Applying the limits of error for  $\bar{X}$ ,  $\pm 3$  times the standard error of estimate, or  $\pm 0.4374$  spore, and carrying it through into the second formula will give the possible range in which the number of spores per cubic centimeter might fall within the precision of the method.

Further work is in progress to determine whether the same accuracy will be obtained by counting a smaller number of fields to obtain the mean number of spores per field from a larger number of smears from sediments.

Since in the experimental work the samples of known spore content contained approximately round numbers of spores—multiples of 50,000—it probably would be sufficiently accurate to designate the number of spores as the nearest multiple of 50,000 to the actual figures derived from the formulas. When using the limits of error  $0 \pm 0.4374$  spore per field, for the estimated mean number of spores per field, it will be found that for numbers below 100,000 there will be some overlapping between 10,000-spore increments, and the value will have to be expressed approximately (for example, the honey contains between 40,000 and 60,000 spores per cubic centimeter); nevertheless the honey can still be designated either as dangerous or as not dangerous.

#### SUMMARY

Previous work on the qualitative demonstration of the presence or absence of spores of *Bacillus larvae* in honey that has been in contact with American foulbrood has been followed by the development of a quantitative method for determining the approximate number of spores per cubic centimeter in such honey. The method is represented by the formula

$$\text{Number of spores per cubic centimeter} = \frac{KNX \times 100 \times D}{N}$$

where  $K$  is the factor for the number of circular fields per 1-cm<sup>2</sup> area,  $N$  is the number of circular fields counted,  $X$  is the actual mean number of spores per field, 100 is the factor that gives the number of spores per cubic centimeter from 0.01 cc of the suspension, and  $D$  is the dilution. The mean number of spores of *Bacillus larvae* per field counted in 60 fields of stained smears made from the sediments obtained by centrifuging 5-cc quantities of honey containing approximately known numbers of spores have been determined by this method.

The mean actual spore count per field was determined for a series of samples of honey prepared to contain approximately 1,000,000, 800,000, 500,000, 300,000, and 50,000 spores per cubic centimeter. The mean theoretical spore count per field that should have been recovered was determined by use of the formula

$$X = \frac{\text{Number of spores per cubic centimeter}}{32,884}$$

The actual mean numbers of spores per field were similar in trend to the calculated theoretical means but were from 10.88 to 15.60 per cent smaller. A statistical analysis of the data to determine the accuracy of the method showed that the calculated probable errors and standard deviations were small. The coefficient of correlation between the actual and the theoretical mean number of spores per field for each sample was found to be  $0.9999 \pm 0.0001$ .

The relation between the actual mean number of spores per field ( $\bar{Y}$ ) and the corresponding most probable values that should have been recovered, estimated from the theoretical mean number of spores per field ( $X$ ), was determined by means of the regression equation  $\bar{Y} = 0.8905X - 0.1791$ . These most probable estimated values were found to be in excellent agreement with the actual values obtained, well within the customary limits of  $\pm 3$  times the standard error of estimate, which was found to be  $\pm 0.1298$  spore.

The most probable theoretical mean number of spores per field ( $\bar{X}$ ) was estimated by means of the regression equation  $\bar{X} = 1.1228Y + 0.2034$ . These values were found to be in excellent agreement with the original calculated values for the theoretical mean, well within  $\pm 3$  times the standard error of estimate,  $\pm 0.1458$  spore.

The statistical analysis of the data therefore indicates that the method used is sufficiently accurate for determining the spore content of unknown samples of honey. For this purpose the following formulas are used:

$$\bar{X} = 1.1228Y + 0.2034 \pm 0.4374$$

where  $Y$  = the actual mean number of spores per field counted from 60 fields, and

$$\text{Number of spores per cubic centimeter} = 32,884\bar{X}.$$

# THE TOXIC EFFECTS OF NAPHTHALENE ON BRUCHUS OBTECTUS AND TENEBRIO MOLITOR IN VARIOUS STAGES OF DEVELOPMENT<sup>1</sup>

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## INTRODUCTION

Although naphthalene has been in use as an insecticide for about 50 years, knowledge of its toxic effects is based mainly on investigations conducted under uncontrolled conditions. Very little is known of the physiological effects of naphthalene on insects. In late years naphthalene has been found useful against an increasing variety of insect pests, but it is probably most effective in killing or repelling insects found in houses, greenhouses, stored products, and in the soil.

The period of exposure necessary to kill the immature forms of the Japanese beetle in a saturated atmosphere of naphthalene depends on the temperature (Fleming and Baker (3)).<sup>2</sup> It ranges from 12 hours at 80° F. to 120 hours at 50°. The relative humidity of the atmosphere also influences insecticidal action on the larvae, mortality increasing with an increase in relative humidity.

Herrick and Griswold (5) found that naphthalene inhibited the development of the eggs of clothes moths, no eggs hatching after an exposure of 14 days in an enclosed space at room temperatures. Hartzell and Wilcoxin (4) observed that naphthalene was toxic to the eggs of the red spider mite. Read (8) showed by laboratory experiments that at least 8 hours' exposure of red spider mite eggs to a saturated atmosphere was required to prevent hatching. A study of the comparative resistance of the larva, protonymph, deutonymph, and adult female to naphthalene vapor showed that there was a slight increase in resistance as the stages advanced from larva to adult. Fleming and Baker (3) found the resistance of immature stages of the Japanese beetle to increase in the following order: (1) larvae, (2) eggs, (3) pupae. It was observed by Mercier (7) that, when the pupae of the fly *Calliphora erythrocephala* Meig. had been exposed to naphthalene vapor, some of the first-generation descendants of the treated flies showed malformations, but in the second generation these malformations did not appear. Shull, Riley, and Richardson (10) found that naphthalene produced no apparent effects on the coagulation of the blood nor on the appearance of the blood cells in the oriental cockroach (*Blatta orientalis* L.): Fleming and Baker (3) observed that eggs and larvae of the Japanese beetle exposed to naphthalene vapor became reddish in color, the intensity of the color depending on the period of exposure. The vapor appeared to have a paralyzing effect on the larvae of the Japanese beetle. Toscano

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<sup>2</sup> Reference is made by number (italic) to Literature Cited, p. 712

Rico (11) also observed the paralyzing effect of naphthalene vapor on the parasitic roundworm (*Ascaris lumbricoides* L.). This paralysis was followed by death.

In view of the more extended use which might be made of naphthalene in the future, a clearer knowledge of the toxic effects of this material would be of much value to entomologists. For this reason investigations on the toxicity of naphthalene to various stages of the bean weevil and yellow mealworm have been conducted under controlled conditions, and the findings are here presented.

## METHODS

The apparatus used in the experiments was a modification of that used by Lehman (6). Humidity was controlled by passing air through saturated solutions of sodium chloride to give the air stream a relative humidity of approximately 70 percent. The apparatus was placed in a chamber the temperature of which was held constant at 25° C. by the use of a bimetallic thermoregulator with a resistance unit as a source of heat. In all experiments air was passed through this apparatus at the rate of 14 liters per hour. To determine whether saturation of the air was obtained, U-tubes containing naphthalene were weighed and placed in the system and then reweighed after the air had passed through them for a definite length of time. The loss in weight of the U-tubes was the amount of naphthalene taken up by the air. The amount volatilizing in a definite volume of air checked closely with the calculations for the amounts of naphthalene in a saturated atmosphere published by Roark and Nelson (9).

Before the beginning of each experiment air was passed over the naphthalene for about one-half hour. The insects were then placed in the flasks and allowed to remain there the desired length of time in contact with a constant stream of air saturated with naphthalene. Flasks containing control insects were similarly treated except that they were not exposed to naphthalene vapor. At the conclusion of each experiment the insects were removed and placed in a rearing chamber at a constant temperature and humidity. All insects in their various stages before and after treatment were kept in the constant-temperature chamber at 25° C. with a relative humidity of approximately 60 percent.

Bean weevils (*Bruchus obtectus* Say) were reared on red kidney beans at a temperature of 25° C. and relative humidity of 60 percent. The cultures were started at weekly intervals so that a new batch of adults might emerge every week.

Larvae of the yellow mealworm (*Tenebrio molitor* L.) were reared on a dry, complete dog food in the form of a meal, while the adults were fed once a week on dog biscuits, canned dog food, and banana skins. The cultures were kept at a constant temperature of 27.5° C. and relative humidity of 70 percent.

## EFFECTS OF NAPHTHALENE ON EGGS OF THE BEAN WEEVIL AND YELLOW MEALWORM

A series of experiments was undertaken to determine the toxicity of naphthalene vapor to the eggs of insects. An attempt was made to determine whether the toxicity varied with the age of the eggs and whether any general physiological effects were produced.

Bean weevil and yellow mealworm eggs, none of which were over 1 day old, were exposed to naphthalene vapor for varying periods of time. The percentage of eggs hatching in the checks was very constant, averaging 90 percent or more (table 1). Naphthalene was toxic to the eggs, the number which failed to hatch varying directly with the period of exposure to the vapor. The large mealworm eggs were affected much more readily when not more than 1 day old than were the small eggs of the bean weevil of the same age. Approximately the same lethal effects were obtained with eggs of the mealworm with only one-half the length of exposure given the bean-weevil eggs.

TABLE 1.—*Toxicity of naphthalene<sup>1</sup> to eggs of the bean weevil and the yellow mealworm at different time exposures*

BEAN-WEEVIL EGGS 0 TO 1 DAY OLD					
Exposure	Trials	Check		Treated	
		Total eggs	Hatched	Total eggs	Hatched
Hours	Number	Number	Percent	Number	Percent
1	3	360	96	1,006	87
2	3	400	91	1,000	80
3	9	1,000	93	2,300	45
4	4	600	91	1,200	14
5	4	500	92	1,300	6
6	4	400	95	1,200	.4
MEALWORM EGGS 0 TO 1 DAY OLD					
¼	2	200	90	600	85
1	3	300	89	700	72
1¼	3	300	89	800	48
2	3	300	91	1,000	25
2¼	3	300	85	900	8
3	5	455	91	735	1

<sup>1</sup> Naphthalene-saturated air stream at 25° C.; relative humidity, 70 percent.

To determine the relation between the stage of development of an insect egg and its susceptibility to the vapor of naphthalene, eggs of both the bean weevil and the mealworm were exposed for 3-hour periods at different ages (table 2). A minimum of 500 eggs was used at each stage of development and several replicates and checks were run with each experiment. The percentage of bean-weevil eggs killed increased with age until the eggs were 4 to 5 days old, when the peak was reached. From that time on toxicity decreased and when the eggs were ready to hatch (6 to 7 days old) very few were killed. The reaction of mealworm eggs to naphthalene vapor was almost the reverse of that of the bean-weevil eggs. The gas was most lethal to eggs 1 day old, with a sudden decrease in toxicity thereafter and a slight increase just before hatching (7 to 8 days old). Newly emerged larvae showed greater resistance than did the eggs to the vapors of naphthalene. These results indicate that the age or stage of development of an insect egg influences its susceptibility to naphthalene.

TABLE 2.—*Age and percentage daily loss in weight of bean weevil and yellow meal-worm eggs in relation to the toxicity of naphthalene vapors*

Age of eggs	Eggs dead after 3-hour exposure		Daily loss in weight of untreated normal eggs		Age of eggs	Eggs dead after 3-hour exposure		Daily loss in weight of untreated normal eggs	
	Bean weevil	Meal-worm	Bean weevil	Meal-worm		Bean weevil	Meal-worm	Bean weevil	Meal-worm
<i>Days</i>	<i>Percent</i>	<i>Percent</i>	<i>Percent</i>	<i>Percent</i>	<i>Days</i>	<i>Percent</i>	<i>Percent</i>	<i>Percent</i>	<i>Percent</i>
0 to 1	55	99	2.72	7.83	5 to 6	68	11	3.48	1.35
1 to 2	70	83	2.71	2.05	6 to 7	22	15	-----	1.68
2 to 3	71	38	2.77	1.51	7 to 8	-----	23	-----	2.04
3 to 4	81	12	3.67	1.18	8 to 9 <sup>1</sup>	-----	0	-----	1.68
4 to 5	90	10	4.56	1.18	9 to 10 <sup>1</sup>	-----	-----	-----	.96

<sup>1</sup> Larvae.

Cotton (2) has shown that the effects of fumigants on insects can be correlated with their respiratory metabolism. During the course of their development the insect eggs which were used in these experiments continuously lost weight, presumably through the loss of water and carbon dioxide. Since both water and carbon dioxide are the end products of respiratory metabolism it is believed that the percentage daily loss in weight of the eggs can be used as a relative measure of the rate of respiratory metabolism.

Normal bean weevil and mealworm eggs were cleaned and weighed on a chemical balance and then placed in closed containers over a saturated solution of ammonium nitrate (60 percent relative humidity) at a temperature of 25° C. The eggs were weighed at 24-hour intervals until they hatched, and the percentage of daily loss in weight obtained (table 2). The data indicate that there is a definite relation between weight loss of the eggs (respiratory metabolism) and the lethal effects of naphthalene. A greater weight loss (respiratory metabolism) was associated with increased mortality of the eggs.

Naphthalene vapor in sublethal dosages prolonged the developmental period of bean weevil eggs. The mean number of days required to complete incubation for bean weevil eggs of different ages which survived exposure for 3 hours to a naphthalene-saturated atmosphere is shown in table 3.

TABLE 3.—*Mean number of days required to complete incubation for bean weevil eggs of different ages which survived 3-hour exposure to a naphthalene-saturated atmosphere*

Age of eggs when treated	Incubation period		Age of eggs when treated	Incubation period	
	Untreated	Treated		Untreated	Treated
<i>Days</i>	<i>Days</i>	<i>Days</i>	<i>Days</i>	<i>Days</i>	<i>Days</i>
1	8.1	9.4	5	7.7	9.1
2	8.0	9.2	6	7.8	8.8
3	7.8	9.1	7	8.0	8.4
4	7.8	9.6			

Exposure in early stages of development delayed hatching of those eggs that survived for more than a day. The greatest delay occurred



when eggs were 3 to 4 days old at about which time naphthalene was most toxic (table 2). When the eggs were ready to hatch (6 to 7 days old) the vapor delayed hatching very little. Those larvae that survived 3-hour exposure to naphthalene were apparently uninjured. The incubation period of the eggs of the mealworm was not significantly affected by the naphthalene treatment. However, the treated eggs, previously creamy white in color, assumed a pink or reddish-brown shade, this color being more prominent in the eggs which had been exposed for the longest period. An examination of the newly emerged larvae from such eggs indicated that this discoloration occurred in the large mid-intestine. No discoloration was observed in other parts of the body as Fleming and Baker (3) had found in larvae of the Japanese beetle. The larvae appeared normal in all other respects and the colored matter disappeared after a few days.

#### EFFECTS OF NAPHTHALENE ON ADVANCED STAGES OF THE YELLOW MEALWORM AND ADULTS OF OTHER SPECIES

Previous data (table 2) showed that after the mealworm eggs had hatched, there was a decided increase in the resistance of the larvae to the lethal effects of naphthalene vapor. Since considerable variation in size was noted among larvae of the same age in the stock cultures, treatments were made with different sized larvae of the same age to determine whether the toxicity of naphthalene vapor was related to the size of the larvae. Larvae of different ages but of the same size were also treated to see whether the age of the larvae affected their susceptibility to naphthalene vapor. After treatment larvae were placed in the constant temperature and humidity cabinet for 48 hours, when the numbers of living and dead insects were recorded. The data (table 4) show that both weight and age influenced larval susceptibility to naphthalene. Sublethal exposures to naphthalene vapor of the newly emerged larvae of the mealworm did not affect subsequent growth as measured by gain in weight.

TABLE 4.—Comparative toxicity of naphthalene to various stages of the yellow mealworm with special reference to size and age of larvae

##### 16-HOUR EXPOSURES

Stage	Age	Treated	Weight (average)	Weight loss (24 hours)	Dead
		Number	Milligrams	Percent	Percent
Larvae.....	2 days.....	50	0.47	5.95	100
	1 month.....	50	1.77	.....	95
	2 months.....	50	1.53	.....	86
	.....do.....	25	8.37	2.62	32
	3 months.....	25	4.85	.....	69
	.....do.....	25	11.32	.....	48
	.....do.....	25	13.47	2.70	32
	.....do.....	25	20.32	2.12	14
	4 months.....	25	5.46	2.71	48
	.....do.....	25	26.00	.....	4

##### 24-HOUR EXPOSURES

Larvae.....	Full grown.....	50	152.76	0.26	20
Prepupae.....	.....	50	.....	2.26	94
Pupae.....	0 to 2 days.....	50	.....	.62	38
	6 to 8 days.....	50	.....	.....	49
	8 to 10 days.....	30	.....	1.19	84.6
Adults.....	0 to 7 days.....	30	.....	2.06	79.6

Full-grown larvae, prepupae, pupae, and adults were exposed to naphthalene vapor for a period of 24 hours (table 4). Forty-eight hours after exposure the larvae and adults were examined and the number of dead recorded. Those unable to crawl were considered as dead. The pupae and prepupae which survived treatments were allowed to develop into adults; those that developed into abnormal adults (undeveloped elytra and pupal abdomens) were recorded as dead. Full-grown larvae were the most resistant to lethal effects of naphthalene vapor and adults were the least resistant. The prepupae showed marked susceptibility to the vapor, whereas the pupae showed marked resistance until a few days before emerging as adults, when their susceptibility increased greatly. It is interesting to note that the stages which lost the greatest weight in 24 hours

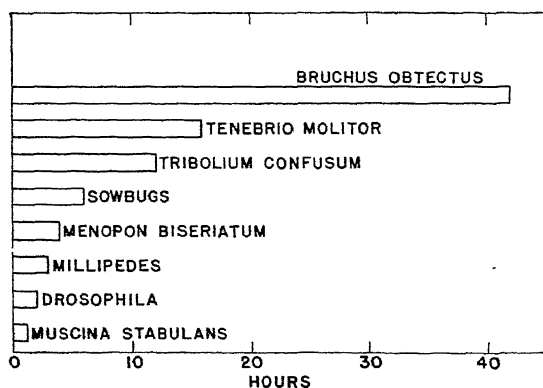


FIGURE 1.—The period of exposure required to kill approximately 100 percent of the species indicated.

were the ones to which naphthalene was the most toxic. About 48 hours after treatment the affected pupae began to turn dark until they became chocolate colored, failed to respond when touched, and finally died. The reaction to naphthalene vapor was very gradual and the nervous and muscular systems were not the first tissues to be affected. Adults emerging from pupae subjected to sublethal dosages produced eggs which were not significantly different in number or fertility from the checks. A 6-hour treatment of adult mealworms had no effect on the fertility of eggs which surviving individuals laid, but it had a decided effect on the number produced. Control specimens produced 5.4 eggs per day per female, while in the treated lot 1.8 eggs per day per female were obtained.

For purposes of comparison several species of adult insects and closely related organisms were exposed to naphthalene vapors for varying periods of time and examined 48 hours after treatment. The time required to kill approximately 100 percent of the insects is shown in figure 1. Diptera were the most susceptible to naphthalene and Coleoptera the least susceptible of the insects used; in general it appeared that the most active insects were the most susceptible.

#### EFFECTS OF NAPHTHALENE INJECTIONS ON YELLOW MEALWORM PUPAE

It was thought that the injection of naphthalene into insects might yield some added information concerning its toxicity. Mealworm pupae were selected for this experiment because they were uniform in size (weight) and were easily handled.

Olive oil was used as a solvent for naphthalene since injection tests with olive oil alone had shown it to be nontoxic to the pupae when injected in much larger amounts than those used with naphthalene. The injection apparatus was similar to that used by Campbell (1). For making injections a rubber tube 8 cm long was attached to the pipette and a screw clamp was attached about 2 cm from the other end. The pipette was filled by suction and the screw clamp tightened. The pupae were held ventral aspect upward and the point of the glass needle was inserted near the lateral margin between the fourth and fifth abdominal segments. The point was directed cephalad for a distance of about 3 mm parallel to the longitudinal axes of the pupae. Pressure was then applied to the rubber tube by the thumb and forefinger which drove the solution into the pupae. The bore of the needle was too small for any blood to enter and the capillary attraction of the small bore prevented the solution from being sucked back when the pressure was released on the rubber tube. After every one or two injections the screw clamp was loosened to release the tension on the liquid in the capillary and then tightened again. The injected pupae were then placed in the constant-temperature cabinet for observation.

Most of the injections were made without visible loss of blood. Pupae up to 2 days of age were used in all the injection experiments. Those that developed into abnormal adults were considered dead. Thirty pupae were used in each experiment. In the check, injected with olive oil alone, 10 percent failed to develop into normal adults. Figure 2 shows the relation between the concentration of naphthalene injected, the death rate, and the percentage of pupae ultimately killed.

The same toxic symptoms appeared in injected pupae as in pupae that were exposed for 24 hours to naphthalene vapor. Within a few days, the time depending upon the amount of material injected, the pupae began to turn dark but still responded to touch by wriggling. They lost all power of movement and were called dead when they finally became chocolate-colored. Examination of the body contents showed discolored plasma, fat, and muscles. All the tissues appeared to be undergoing disintegration. Naphthalene injected into the pupae did not produce materially different effects from those obtained with vapors.

From these injection experiments it seems that naphthalene vapor to be effective must be absorbed by the body tissues; that it is a

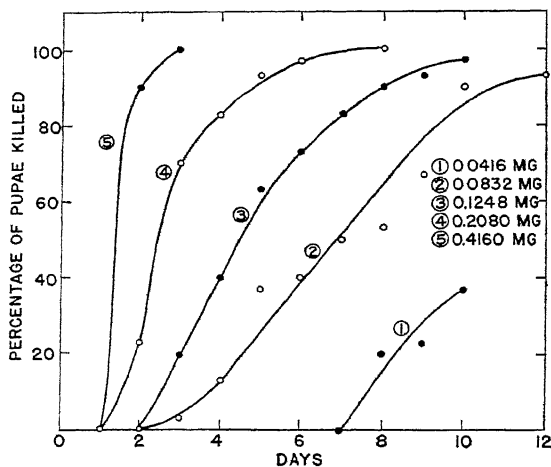


FIGURE 2.—The relation between the concentration of naphthalene injected into mealworm pupae, the rate of kill, and the total percentage of pupae killed.

slow-acting poison to the pupae of the mealworm; and that it affects other tissues before it affects the nervous or muscular systems. The fat bodies were probably the first tissues to be affected since they appeared to be partly disintegrated and discolored in treated larvae and pupae.

#### SUMMARY AND CONCLUSIONS

A study of the toxicity of naphthalene to the bean weevil (*Bruchus obtectus* Say) and the yellow mealworm (*Tenebrio molitor* L.) in their various stages of development is reported.

Experiments with the eggs of the bean weevil and the mealworm showed that toxicity varied with the age of the eggs. It was found that the rate of respiratory metabolism of the eggs measured by the percentage daily loss in weight was directly related to lethal dosages of naphthalene. An increased loss in weight was associated with a greater toxicity of naphthalene to the eggs. The development of bean weevil eggs was noticeably retarded by sublethal exposure to naphthalene vapor. The developmental rate of mealworm eggs was not significantly affected by sublethal exposures to naphthalene. Eggs and very young larvae of the mealworm when fumigated with naphthalene turned reddish brown, the color intensity varying directly with the period of exposure.

Experiments with larvae of the mealworm showed that the toxicity of naphthalene decreased with increased age and weight of the larvae. Sublethal exposures of mealworm eggs or young larvae to naphthalene did not affect subsequent growth.

Resistance to naphthalene varied greatly between instars of the more advanced stages of the mealworm. The order of susceptibility of all the stages of the mealworm to naphthalene from least to greatest resistance was: (1) Eggs, (2) young larvae, (3) adults, (4) prepupae, (5) pupae, (6) mature larvae. Increased respiratory metabolism as measured by percentage daily loss in weight appeared to be related to increased toxicity of naphthalene with the different stages of the mealworm.

In experiments with several species of adult insects and closely related organisms Diptera were most susceptible to naphthalene while Coleoptera were most resistant.

Injections of olive-oil solutions of naphthalene into mealworm pupae produced the same toxic effects as when the pupae were exposed to naphthalene vapor. Naphthalene was a slow-acting poison to the pupae even when injected into the body. The nervous and muscular systems were not the first tissues to be affected, as shown by the wriggling movements of darkened, dying pupae when touched. Fat bodies were probably the first tissues to be affected since they appeared to be partly disintegrated and discolored in treated larvae and pupae.

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# SOME ENVIRONMENTAL FACTORS INFLUENCING THE DEVELOPMENT OF HAIRY ROOT ON APPLE<sup>1</sup>

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## INTRODUCTION

The influence of certain environmental factors on the development of hairy root has been studied under partially controlled conditions. The need for such studies was indicated in a report by Riker and Hildebrand<sup>2</sup> on the seasonal development of hairy root on nursery apple trees grown from piece-root grafts. Various factors for infection and the development of disease appeared to be less favorable either at the beginning or at the end of the season than at midseason. Consequently, it appeared desirable to examine the relative importance of various soil temperatures and moistures when other factors were similar. Correlated with this work the effect of temperature on the growth of the causal organism, *Phytophthora rhizogenes* R. B. W. K. and S., was determined.

The objects of the present study were to measure the influence (1) of various soil temperatures on the development of hairy root, (2) of various soil moistures on the development of hairy root, and (3) of temperature on the causal organism.

## MATERIALS AND METHODS

The host plants in these studies were 1-year-old root-grafted Fameuse apple trees, grown in southern nurseries and selected for uniformity. The trees varied in caliper from five-sixteenths to three-eighths of an inch and in height from 3 to 4 feet from the graft union. Before they were planted the roots were trimmed until they would fit into the containers used, and the tops were cut off at approximately 2 feet from the crown. As the trees grew, no more than two shoots were permitted to develop.

The experiments were all conducted in the greenhouse during the late winter and early spring months. This was the only time of the year when dormant trees could be planted and handled satisfactorily over a sufficient period of time at the desired soil temperatures.

The containers in which the trees were grown were cylindrical galvanized-iron cans 8 inches in diameter and 16 inches high. Two trees were planted in each can.

The soil moisture was regulated in the usual way by adding water until the lost weight was restored. The water was added both at the surface and through perforated aluminum tubes placed vertically in the middle of the cans. The sandy loam employed had a moisture-holding capacity approximating 40 percent of its dry weight. The

<sup>1</sup> Received for publication Dec. 16, 1935; issued June 1936. This work was correlated with a project carried on cooperatively between the Bureau of Plant Industry, U. S. Department of Agriculture, and the University of Wisconsin. It was supported in part by a grant from the special research fund of the University of Wisconsin. See legend fig. 1.

<sup>2</sup> RIKER, A. J., and HILDEBRAND, E. M. SEASONAL DEVELOPMENT OF HAIRY ROOT, CROWN GALL, AND WOUND OVERGROWTH ON APPLE TREES IN THE NURSERY. *JOUR. Agr. Research* 48: 837-812, illus. 1964.

determinations were all made in percentages of the moisture-holding capacity of the soil. The various trials on soil moisture were made in a greenhouse where the temperature averaged about 24° C.

The soil temperature was regulated by placing the cans in Wisconsin soil-temperature tanks and adjusting the temperature of the water. The temperatures in the soil employed were 12°, 16°, 20°, 24°, 28°, and 32° C. The surface of the soil in every case was covered with an inch of ground cork to lessen evaporation and to maintain more uniform temperatures in the upper layers of soil. The soil moisture in the temperature series was 80 percent of the moisture-holding capacity of the soil in 1931, but was 75 percent in the later three trials. The tanks employed and the general procedure, except as noted, were similar to those used earlier by Riker.<sup>3</sup>

The cultures employed in these studies were the progeny of individual cells. Their previous history has been described by Wright et al.<sup>4</sup> under the numbers C-10, C-11, and C-12. They were introduced through scalpel cuts into the scions of the young apple trees after they had broken dormancy. The five inoculations in each tree were made well below the surface of the soil.

Three trials involving soil-temperature and soil-moisture series were run, respectively, during 1931, 1932, and 1933. Since difficulty was experienced with the 32° C. tank in 1931, and the temperature 28° had previously been found suitable and 32° unsuitable for the formation of crown gall on tomato,<sup>3</sup> particular attention was paid to these two temperatures in trials during 1934.

The numbers employed in the individual temperature trials were uniform. For each soil temperature during every season a single trial included eight cans with two trees each and five inoculations per tree. Thus every trial was based upon 80 inoculations. In each of the soil moistures employed four cans were used in 1933 and six cans in 1931, 1932, and 1934.

#### DEVELOPMENT OF HAIRY ROOT AT VARIOUS SOIL TEMPERATURES AND MOISTURES

The results of these studies were recorded in both the temperature and moisture series on (1) the growth in height of the host plant, (2) the percentage of infection by the hairy root bacteria, and (3) the growth measured by weight of the hairy root at the point of inoculation.

The records of the different trials in 1931, 1932, and 1933 have been averaged to secure the data presented. In 1931 no infection was secured in the 32° tank, which correspondingly lowered the final averages for incidence of infection and for weight of hairy root. In 1934 three tanks containing eight cans with two trees each were run at approximately 28° C. A similar set was run at 32°. Since these results corroborated the data presented at these temperatures, the details are omitted.

The growth of the host plant varied over the temperature range employed (figs. 1 and 2, A). It was greatest at 20° C., relatively

<sup>3</sup> RIKER, A. J. STUDIES ON THE INFLUENCE OF SOME ENVIRONMENTAL FACTORS ON THE DEVELOPMENT OF CROWN GALL. *Jour. Agr. Research* 32: 83-96, illus. 1926

<sup>4</sup> WRIGHT, W. H., HENDRICKSON, A. A., and RIKER, A. J. STUDIES ON THE PROGENY OF SINGLE-CELL ISOLATIONS FROM THE HAIRY-ROOT AND CROWN-GALL ORGANISMS. *Jour. Agr. Research* 41: 541-547, illus 1930



vigorous at 16°, 24°, and 28°, and relatively poor at 12° and 32°. The growth of the host plant in the different soil moistures employed in 1932, 1933, and 1934 appeared to be approximately the same at 75 and 90 percent of the moisture-holding capacity, but was distinctly lower at 60 percent (fig. 3). The growth during 1931 in a trial with 40 percent moisture was quite inferior. The number of successful inoculations and the weight of hairy roots were also less. Other trials in 1931 at 60- and 80-percent moisture gave results comparable to those at 60- and 75-percent moisture during 1932, 1933, and 1934.

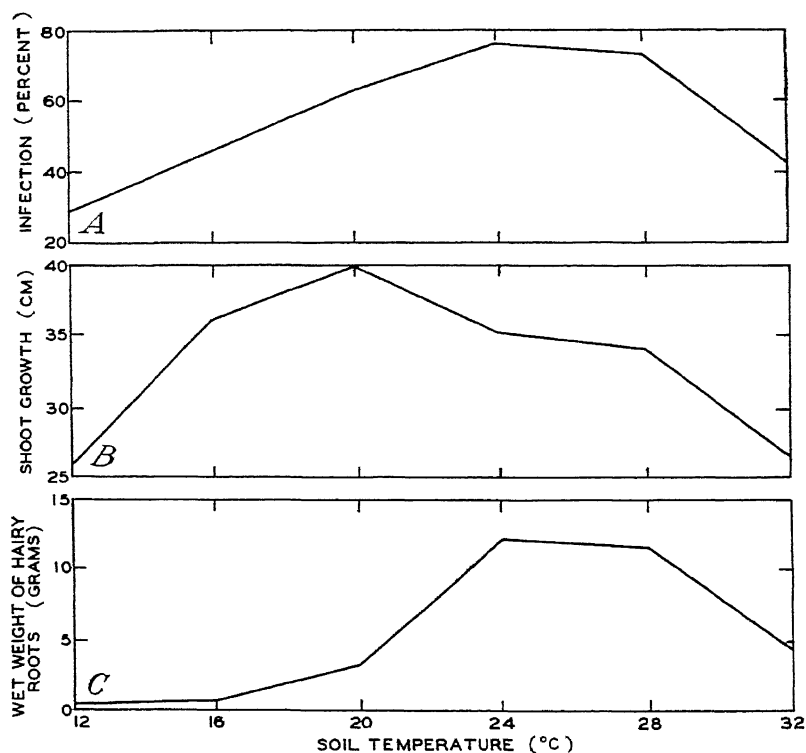


FIGURE 1.—The effect of soil temperature. *A*, On the percentage of infection on young apple trees by hairy root bacteria; *B*, on the length of shoot growth of the apple trees, *C*, on the amount of hairy root growth as measured by wet weight. All illustrations prepared by Eugene Herling.

The percentage of infection by the hairy root bacteria was greatest at 24° and 28° C., and progressively less above and below these temperatures (fig. 1). The percentage of infection was slightly but not significantly greater at 75 percent of the moisture-holding capacity of the soil than at either 60 or 90 percent (fig. 3).

The growth of hairy root (fig. 2, *B*) following infection, as measured by wet weight approximately 10 to 11 weeks after inoculation, was greatest at 24° and 28° C., but progressively less at higher and lower temperatures (fig. 1). The growth of hairy root in the moisture series very closely paralleled that for growth of shoots. It was

relatively good from 60 to 90 percent; being approximately the same at 75 and 90 percent and slightly less at 60 percent (fig. 3). The development of hairy root, like that of crown gall<sup>5</sup>, seems to be associated with the vigor of the host growth.



FIGURE 2.—A, shoot growth of young apple trees at soil temperatures of 12°, 16°, 20°, 24°, 28°, and 32° C. from left to right, respectively; B, hairy root growth at the same temperatures.

#### GROWTH OF HAIRY ROOT BACTERIA AT DIFFERENT TEMPERATURES

The growth of the hairy root bacteria was studied in both solid and liquid media at temperatures ranging, at 4° intervals, from 4° to 36° C., inclusive. Needle-point transfers were made to plates poured with carrot-extract agar so that seven equidistant colonies were grown in each Petri dish. Five dishes were grown at each temperature. The average diameters of these colonies after 16 days were taken as

<sup>5</sup> RIFER, A. J. See footnote 3.

the measure of growth at the different temperatures. The trials were made three separate times and all results averaged. Thus each point on the graph (fig. 4) represents the average diameter of 105 colonies.

Similar trials were made with yeast-infusion mannite agar that had the following composition: Magnesium sulphate ( $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ ), 0.2 g; sodium chloride ( $\text{NaCl}$ ), 0.2 g; calcium chloride ( $\text{CaCl}_2$ ), 0.1 g; dibasic potassium phosphate ( $\text{K}_2\text{HPO}_4$ ), 0.2 g; 10 percent yeast infusion, 100 cc; mannite, 10 g; agar, 17 g; and water to make 1 liter.

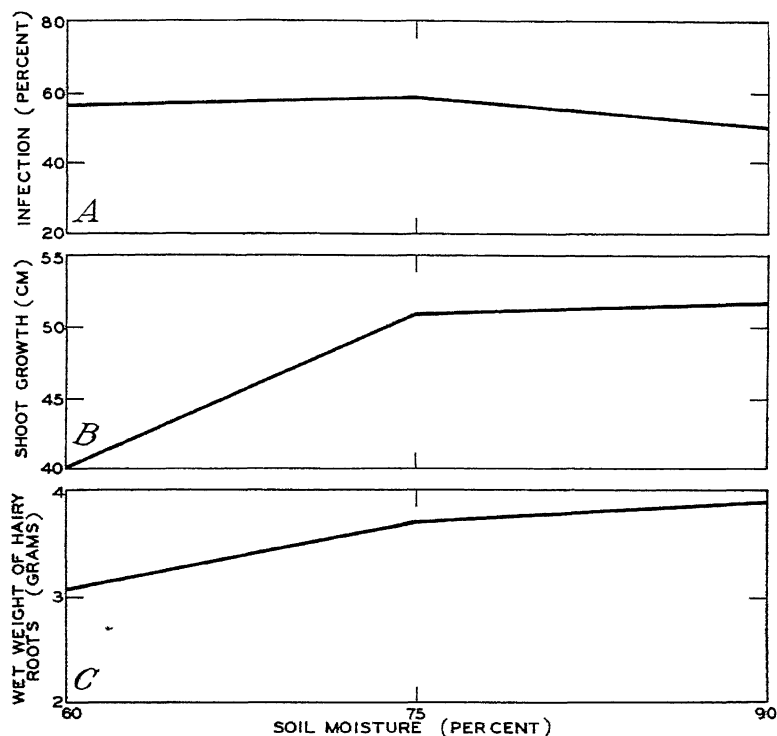


FIGURE 3.—The effect of soil moisture: A, On the percentage of infection on young apple trees by hairy root bacteria; B, on the length of shoot growth of the apple trees; C, on the amount of the hairy root growth as measured by wet weight.

The reaction was adjusted to pH 7.0. The results are shown in figure 4.

Similar trials were made with the yeast-infusion mannite media described above except that no agar was added. The amount of growth was measured by turbidity in relation to the McFarland <sup>6</sup> nephelometer scale. The results appear in figure 4.

These three different trials show that the largest amount of vegetative growth occurred at approximately 28°, although the growth was relatively good between 20° and 32° C. A slight amount of growth was found at both 4° and 36°.

<sup>6</sup> MCFARLAND, J. THE NEPHELOMETER: AN INSTRUMENT FOR ESTIMATING THE NUMBER OF BACTERIA IN SUSPENSIONS USED FOR CALCULATING THE OPSONIC INDEX AND FOR VACCINES. *JOUR. AMER. MED. ASSOC.* 49: 1176-1178, illus. 1907.

## DISCUSSION

These studies assist in the interpretation of several results previously reported by Riker and Hildebrand<sup>7</sup>, particularly in relation to the incidence of infection, incubation period, and rate of growth of apple trees and hairy roots.

The percentage of infection was greatest at 24° and almost as great at 28° C. This is at the upper limit for vigorous growth of the trees and is approximately at the optimum for the growth of the bacteria.

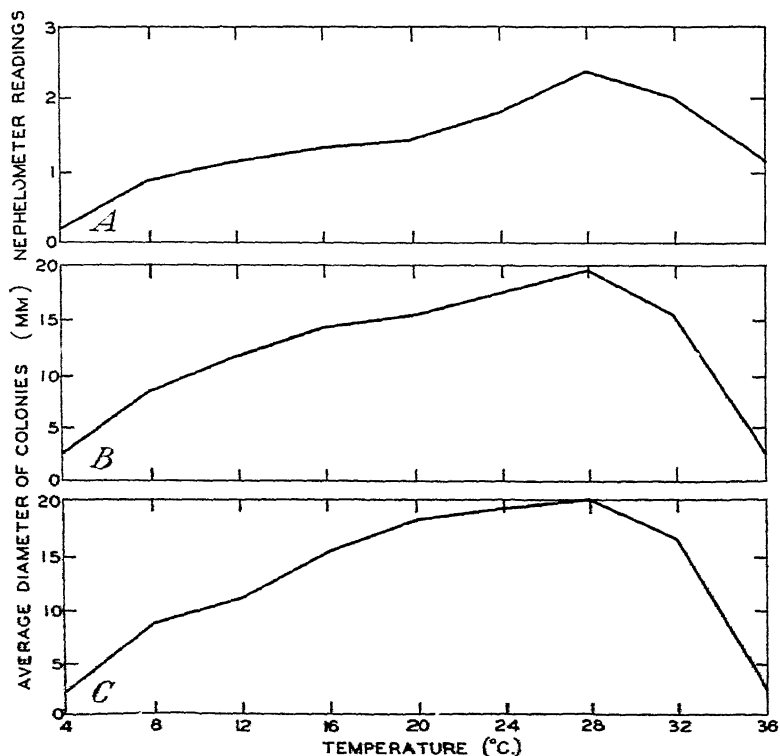


FIGURE 4.—The effect of temperature on the development of hairy root bacteria, as measured (A) by turbidity in tubes of liquid yeast-infusion mannite media and by the average colony diameters on (B) yeast-infusion mannite and on (C) carrot-extract agars

The smaller percentage of infection at lower temperatures correlates with the low incidence of infection in the spring noted by Riker and Hildebrand when the apple grafts were inoculated at the time of grafting. However, these writers found that by midsummer the incidence of infection was very high. It seems likely from the symptoms of the hairy root observed at the lower temperatures that a much higher incidence of infection would have occurred if the inoculated trees either had been given a longer time or had been exposed to a higher temperature. Perhaps a similar explanation would account for the variation in the percentage of infection with varying soil

<sup>7</sup> RIKER, A. J., and HILDEBRAND, E. M. See footnote 2.

moisture; i. e., the incubation period was not long enough under the conditions to give the maximum percentage of infection.

The incubation periods defined and observed by Riker and Hildebrand were shortest during midsummer when the temperature was high and the trees were growing rapidly. Thus the environmental conditions that favor short incubation periods appear also to favor the growth of hairy roots.

The active development of hairy root was greatest at 24° and 28° C., temperatures at which the trees were growing vigorously and the bacteria were making maximum vegetative growth. Thus it appears that the short incubation periods are correlated with conditions favorable for active growth by both host and bacteria.

The growth of the apple shoots was relatively vigorous between 16° and 28° C., inclusive. Although the growth of both the host and the bacteria was relatively vigorous at 16° and 20°, the hairy roots grew more actively at the higher temperatures. No reason for this is apparent. The question might be raised whether the factors for root stimulation are present in larger quantity in this 24° to 28° range. In the moisture series the growth of the hairy root appears to be correlated with that of the host.

#### SUMMARY

Inoculations with *Phytophthora rhizogenes* were made on 1-year-old Fameuse apple trees kept at graduated soil temperatures and soil moistures. The highest incidence of infection, in the time of the experiment, appeared at 24° to 28° C., and at 60 and 75 percent of the moisture-holding capacity of the soil. This probably indicated the shortest incubation period. The growth of the shoots in length was greatest at 20°, with vigorous growth between 16° and 28°. Their growth was also more vigorous at 75 and 90 percent soil moisture than at 60 percent. The development in weight of hairy root was greatest at 24° to 28°. It was good over the entire range and only slightly greater at 75 and 90 percent moisture than at 60 percent. The air temperature was approximately 24°.

The growth of the hairy root bacteria was measured by colony diameter on yeast-infusion and carrot agar, and by turbidity in a yeast-infusion liquid medium, at temperatures from 4° to 36° C. Vegetative growth was greatest at 28°, good at 16°, 20°, 24°, and 32°, moderate at 8° and 12°, and slight at 4° and 36°.



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## PHYSIOLOGICAL CHANGES IN THE RIND OF CALIFORNIA ORANGES DURING GROWTH AND STORAGE<sup>1</sup>

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### INTRODUCTION

This paper deals with the changes that normally occur in the rind of Valencia and Washington Navel oranges (*Citrus sinensis* (L.) Osbeck) both while the fruit is on the tree and after it is picked and held in storage at various temperatures. The investigation formed part of a general physiological study of the rind of citrus fruits with particular reference to the cause or causes of certain rind pitting and spotting diseases that occur in transportation and storage.

When the work was begun it was deemed desirable to place the greater emphasis upon the determination of the normal changes in the rind that occur while the fruit is on the tree. However, even at that time, a few collections were taken for storage study. Emphasis was not shifted to the determination of changes in the rind under various storage conditions until considerably later. Therefore, this paper is concerned almost entirely with the rind of Valencia and Washington Navel oranges in the field, and the observations on the effects of cold storage are to be considered preliminary.

Although the results of this investigation allow the comparison of the Washington Navel and Valencia orange rinds in certain aspects, it should be kept in mind that under the circumstances much more than varietal characteristics must be considered. One of the most important factors involved is the difference in the season of the year in which the two varieties are harvested. This phase of the investigation is considered in the section of this paper dealing with a comparison of the Washington Navel and the Valencia orange rinds.

### REVIEW OF LITERATURE

In previous papers on oranges a preponderant amount of attention has been given to the pulp and juice. The reason for this is readily understood, but when one comes to deal with an important disease that has its seat entirely within the rind, the physiology of this tissue assumes a new interest. It is true that much is known regarding the essential oils of the rind, but relatively little is known of its other constituents, particularly in relation to their seasonal variations in the field or in storage.

Bigelow and Gore (1)<sup>2</sup> observed changes in the proportion of rind to pulp of oranges during ripening, and later Copeman (3) made

<sup>1</sup> Received for publication Aug. 30, 1935; issued June 1936.

<sup>2</sup> Reference is made by number (italic) to Literature Cited, p. 746.

similar observations through a period of 104 days. Both of these papers record a small but constant decrease in the proportion of rind to pulp as the season advances. Willimott and Wokes (8, 9) reported an extensive study of seasonal distribution of vitamins, oxidases, sugars, and acid in the pulp and rind of citrus fruit. Recently Gaddum (4) has followed the seasonal variation of the pectins in Valencia oranges. He found that the pectic compounds remained practically constant in the albedo and pulp until after the full-ripe stage, when they began to decline; and that the water-soluble pectins increased to a maximum just previous to the beginning of the decline of the pectic compounds, after which they also began to decline—a situation which is of interest in connection with the late-season increase of susceptibility to storage spot often observed. Many other papers are concerned either wholly or in part with the orange rind, but most of the experiments reported were not designed to show seasonal behavior. As a guide to these works, the extensive bibliography of the genus *Citrus* compiled by Matlack<sup>3</sup> is recommended.

#### MATERIALS AND METHODS

The study reported herein was carried on through the orange seasons of 1932 and 1933 in two localities, Pomona and Corona, Calif.<sup>4</sup>

Collections could not be made from the two localities on the same day because of the time required in the laboratory to give each the necessary immediate care. Usually the Corona collection was made 1 day after the Pomona collection, but in December and January there was a difference of 4 days; and in both instances a rise of temperature occurred immediately following the collection at Pomona. As a result the means for the 5-day periods preceding the corresponding collections at Corona were several degrees higher than they would have been had the pickings been made the next day after the ones at Pomona.

Collections were always made in the early morning. Each field collection consisted of 50 fruits, or 10 from each of 5 selected trees, taken with as uniform spacing as possible. When the fruit arrived in the laboratory the rinds were removed at once and each rind was divided into six portions as follows: (1) Stem-end flavedo;<sup>5</sup> (2) stem-end albedo, (3) equatorial flavedo, (4) equatorial albedo; (5) blossom-end flavedo, and (6) blossom-end albedo. A separation of this sort was considered desirable on account of the frequent localization, especially in the Valencia orange, of pitting and spotting in more or less specific portions of the rind. This arrangement allowed the making of four major comparisons, namely: (1) Seasonal, as regards the rind as a whole, and both general and seasonal of (2) stem and blossom ends, (3) flavedo and albedo, and (4) fruit from the two localities. The two equatorial portions were analyzed exactly as were the stem-end and blossom-end portions, but the data are not considered necessary for this report and are not presented, unless specifically mentioned. The quantities recorded for the equatorial portions are almost always intermediate in value between the corresponding ones in the stem and blossom ends.

<sup>3</sup> MATLACK, M. B. BIBLIOGRAPHY ON THE CHEMISTRY OF THE GENUS *CITRUS*. U. S. Dept. Agr., Bur. Chem. and Soils. 159 pp. 1932. [Mimeographed.]

<sup>4</sup> These two localities are about 30 miles apart and in the same general district. The grove used in Corona was at the southeastern side, and the one in Pomona was near the northern side of the district. The climate was approximately the same for each grove, although at Corona the temperatures were somewhat higher and there was more sunshine and less humidity.

<sup>5</sup> The terms "flavedo" and "albedo" designate outer rind and inner rind, respectively.



Water was determined in fresh lots which were separated from the general macrochemical sample just previous to the preservation of the latter in alcohol. Two methods were employed: (1) The usual drying of the tissue to constant weight and (2) the toluene method. The latter method, in spite of its known faults, was found to be the more satisfactory for the flavedo because of the error introduced by oven drying, due to the high volatile-oil content of this outer rind tissue.

The general macrochemical samples consisted of 50 g fresh weight, and these, as just indicated, were preserved by covering them with 95-percent alcohol and heating to 70° C. for 1 hour.

Nitrogen and carbohydrates were determined by the methods described by Harvey (6, pp. 8, 9).

The sugars were calculated as follows: (1) "Reducing sugar", as glucose, from the copper equivalent to the permanganate titration before inversion; (2) "invert sugar", as invert sugar, from the copper equivalent to the permanganate titration after inversion minus the permanganate titration before inversion; and (3) "total sugar", as the sum of "reducing sugar" plus "invert sugar."

Hesperidin was determined in the rind of the Washington Navel variety only. The method of analysis was as follows: Into a 300-ml Florence flask were transferred 25 ml of the extract, or soluble fraction, 51 ml of water, and 4 ml of concentrated hydrochloric acid. This mixture was allowed to simmer for 40 minutes under a reflux condenser. The contents of the flask were then cooled, neutralized, and filtered.

In the early analyses hesperidin was determined by two methods: (1) By collecting the insoluble derivative, hesperitin, on a Gooch filter, then drying and weighing; and (2) by determining the increased reducing power after hydrolysis. Both methods were tested with pure hesperidin solutions as well as with tissue extracts. The gravimetric method, although satisfactory otherwise, required such large samples (200 ml of extract) to obtain sufficient accuracy that it was finally abandoned in favor of the other method, that is, the determination of the sugars derived from hesperidin. The weight of the sugars found was multiplied by the factor 2.17. This is larger than the theoretical factor, but it was found that the destruction of sugar during hydrolysis made this modification necessary.

Hydrogen-ion determinations were made on the rind of Washington Navel oranges by means of a Leeds and Northrup "student" potentiometer with a quinhydrone electrode. Juice from the different rind portions was obtained by means of a specially constructed press after the tissue had been frozen for 24 hours at temperatures of -15° to -20° F. Flavedo tissue readily yielded abundant juice, but it was often difficult to express sufficient quantities of juice from the amount of albedo tissue available.

In making the hydrogen-ion determination it was found that the potential of the rind juice showed a strong tendency to drift during readings. This difficulty was largely overcome by using a large excess of quinhydrone and by diluting the juice to five times its original volume. At this dilution the pH was approximately the same as that of the undiluted juice, whereas a lesser dilution had a tendency to give a smaller pH and a greater dilution a larger pH than the original.

In each locality five adjacent trees of Valencia oranges were selected to furnish the experimental material. The trees at Pomona were 28 years old and were growing in Hanford gravely sandy loam. One

half of each tree was irrigated every 2 weeks. At Corona the trees were within a year of the same age and were growing in Yolo gravelly loam. The whole of each tree was irrigated about every 30 days.

From May 18 to November 14, 10 collections of fruit were made from each locality. The results indicated later that the first collection probably should have been made about May 1 or April 15 instead of May 18 when the fruit was already well colored. The interval between collections was 2 weeks except the last three intervals, which were approximately 30 days. In November additional lots were collected from each locality and stored, some at 33° and some at 53° F. These were held for 7 weeks for observation and analysis.

The Washington Navel oranges were from similarly selected trees in the same localities. The trees in the Pomona grove were 43 years old and those in the Corona grove were 30 years old.

The intervals between collections varied with the season as follows: Those between collections nos. 1 and 2, and 2 and 3 were 3 weeks; those between nos. 3 and 4, and 4 and 5 were 4 weeks; and the interval between nos. 5 and 6 was 6 weeks.

Additional lots of fruit were collected from each locality for storage at 52°, 42°, and 32° F. These collections corresponded with the field collections of January (no. 3), March (no. 5), April (no. 6), and the extra Pomona field collection.

Six regular field collections were made during a period of 140 days beginning November 25, 1932, and ending April 13, 1933. The first collection was made before the usual commercial picking time, when the fruit was still dark green, and the last was made after the regular picking season. From the first the Pomona fruit was somewhat deeper in color than the Corona fruit, and as the season advanced it became evident that the latter was maturing more rapidly than the former. By the first week in January the fruit at both locations was well "colored." One extra field collection was made at Pomona on May 25 to serve as a control for a storage experiment that was started on that day. Since the last collection date at Corona coincided with the time of full bloom, the fruit was falling readily and showed some lack of firmness. On the corresponding collection date at Pomona the fruit was still firm, and the trees were not in full bloom until May 25. At the last, or extra, collection at Pomona the trees were blossoming and the fruit was much like that at Corona on the previous collection date.

## EXPERIMENTAL RESULTS

Most of the experimental data reported herein were obtained under field conditions, but some were obtained under conditions of storage.

### CHANGES UNDER FIELD CONDITIONS

#### SEASONAL CHANGES

The chemical data are summarized in table 1, which gives the average values of the different substances under observation. These averages were calculated from the combined results of all portions of the rind and from both localities for each collection date; for Valencias each value is the average of 24 analyses and for Washington Navels it is the average of 16 similar analyses.<sup>6</sup>

<sup>6</sup> Duplicate samples were analyzed from each of the two localities; with the Valencia rinds there were six portions, but with the Washington Navels only four portions since the equatorial portions were not analyzed.

TABLE 1.—Seasonal changes in the chemical composition of orange rinds<sup>1</sup>

Variety	Date of collection <sup>2</sup>	Reducing sugar	Invert sugar	Total sugar	Soluble solids	In-soluble solids	Hydrolyzable polysaccharides	Soluble nitrogen	In-soluble nitrogen	Total nitrogen	Water	Flavonoid-albedo ratio	Hesperidin	Hydrogen-ion concentration
		Percent	Percent	Percent	Percent	Percent	Percent	Percent	Percent	Percent	Percent		Percent	pH <sup>3</sup>
Valencia	1935													
	May 21	20.11	9.18	29.29	48.57	41.36	13.63	0.384	0.466	0.850	72.0			
	June 4	21.09	8.78	31.44	54.35	38.09	11.92	.392	.444	.836	72.4	1.57		
	June 18	21.86	10.07	31.43	53.57	37.89	12.96	.379	.461	.840	72.0	1.57		
	July 2	20.37	10.96	31.03	52.75	37.73	12.71	.405	.477	.882	72.7	1.59		
	July 16	18.06	10.18	29.24	51.62	36.82	12.20	.404	.468	.852	72.3	1.63		
	July 30	18.49	11.99	31.14	51.84	38.74	13.08	.435	.484	.919	72.8	1.60		
	Aug. 13	18.63	11.92	30.90	51.12	37.61	12.43	.426	.468	.894	73.2	1.66		
	Sept. 10	17.95	13.85	28.00	50.77	38.42	13.23	.456	.495	.951	74.0	1.77		
	Oct. 8	17.28	14.68	28.34	51.68	38.35	11.49	.463	.465	.928	74.8	1.68		
	Nov. 12	20.74	7.71	28.45	52.80	37.01	15.35	.426	.430	.856	72.0	1.73		
	Nov. 25	24.02	5.71	29.73	52.99	47.01	15.35				75.0	1.03	3.45	5.38
	Dec. 16	27.93	6.09	34.02	57.47	42.53	13.51				74.5	1.04	3.70	5.62
Washington Navel	1935													
	Jan. 6	30.37	4.76	35.13	56.49	43.51	14.22				72.2	1.12	3.07	5.61
	Feb. 1	31.56	6.63	38.19	60.17	39.83	13.28				73.1	1.02	2.91	5.53
	Mar. 1	34.20	6.35	40.55	61.60	38.01	12.55				72.4	1.02	2.90	5.89
	Apr. 12	34.85	6.27	41.12	62.05	37.05	11.93				74.6	1.02	2.63	5.73

<sup>1</sup> All values except water and hydrogen-ion concentration are calculated on the basis of dry weight.<sup>2</sup> Collections in the 2 localities were 3 days apart, the Pomona collection always preceding the Corona.<sup>3</sup> The writers are fully aware that the averaging of pH values, here and elsewhere in this paper, is mathematically unsound, but they believe that through the ranges shown the averages of pH values as numerical entities are probably as sound biologically as the averages of the actual hydrogen-ion concentrations or the equivalent pH values, and are considerably more convenient.

**Water.**—In such a summary of data as that given in table 1 it does not appear at a glance that there was really a seasonal trend of water content; but on closer examination and in consideration of the number of analyses represented, it becomes clear that in Valencia oranges there was a gradual rise of water nearly to the end of the season (fig. 1). After that time there was a slight decrease of water, as recorded in the last collection. In the data from individual portions of the rinds (data which cannot be presented here on account of limited space) some considerable fluctuations of water were noted. However, all these larger variations seemed to be correlated chiefly with the availability of soil moisture, although there was some correlation with temperature and other environmental factors, but not with the

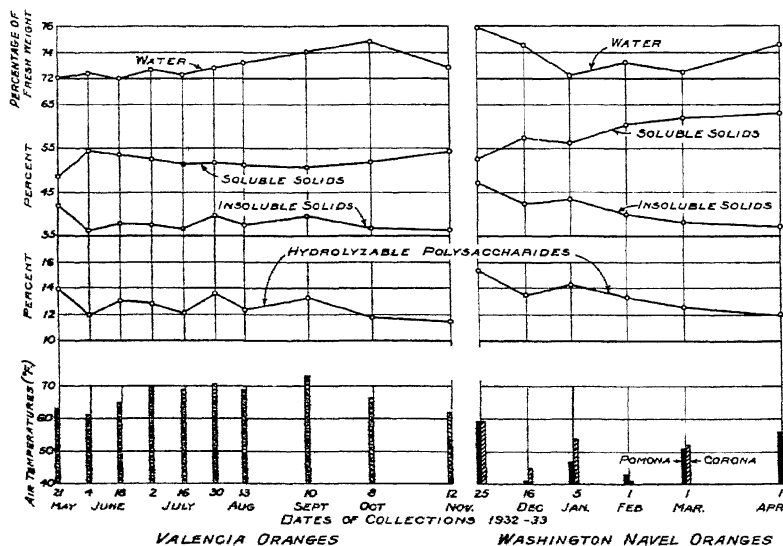


FIGURE 1.—Seasonal changes in mean air temperature (means for 5-day periods preceding collections), water, soluble and insoluble solids, and hydrolyzable polysaccharides content of the rind of Valencia and Washington Navel oranges.

maturity of the fruit. That is to say, the water-holding power of orange rinds changed only slightly with the season. The water content was not correlated closely with mean temperature, possibly because all collections were made early in the morning, when the saturation deficit set up during the previous afternoon may have been mostly satisfied.

The magnitude of this possible diurnal variation of water content in Valencia rinds was investigated through a period of 24 hours. Six collections were made during a night and day following a hot day. The largest diurnal difference between midafternoon and early morning was about 2 percent, but this value is probably not very close to the maximum variation produced by the occasional severe conditions encountered in orange groves during the summer.

In Washington Navel oranges the water content showed a downward trend during the early part of the season, followed by fluctuations due to immediate environmental factors (fig. 1).

**Soluble solids.**—In Valencia oranges, as shown in figure 1, the soluble solids in the rinds as a whole vary almost inversely with the mean temperature.<sup>7</sup> There is a broad minimum during August-September, corresponding to the hottest weather of the season, and two maxima, one in June and the other in October-November, the coolest periods. The separate portions of the rinds often showed greater fluctuation but in the same direction. The higher temperatures and the lower water content evidently favored the condensation of sugars in the rinds to insoluble polysaccharides, hence the lower soluble solids. In Washington Navel oranges, on the other hand,

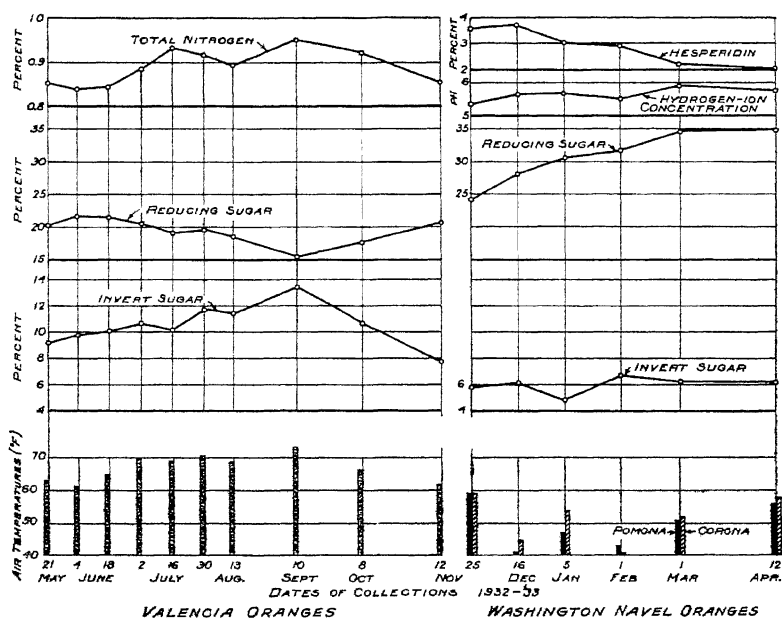


FIGURE 2.—Seasonal changes in mean air temperature (means for 5 day periods preceding collections), reducing and invert sugar, total nitrogen, hydrogen-ion concentration, and hesperidin content of the rind of Valencia and Washington Navel oranges.

there was an increase in soluble solids and a decrease in insoluble solids as the season advanced.

**Hydrolyzable polysaccharides.**—In Valencias the polysaccharides graph (fig. 1) shows a strong tendency to parallel the mean-temperature graph. When temperatures were high the polysaccharides were high, and vice versa. Individual portions of the rind showed this tendency more strikingly than did the rind as a whole. In Washington navels the graph for polysaccharides practically parallels that for insoluble solids.

**Total sugar.**—In Valencia oranges total sugar showed little change through the season, owing to the fact that the behavior of the reducing sugar was almost the reverse of that of invert sugar on all

<sup>7</sup> The mean-temperature graph was constructed by averaging the mean temperature of the 5 days immediately preceding each collection date at each locality. The two graphs resulting were essentially alike throughout, that of Corona being somewhat the higher.

occasions: if reducing sugar was high, invert sugar was low, and vice versa. The graph in figure 2 representing reducing sugar is somewhat similar to that of soluble solids (fig. 1). The invert-sugar graph (fig. 2), on the other hand, nearly parallels the mean-temperature graph.

The seasonal trend of reducing sugar is of considerable interest. There are two maxima and one minimum. The first maximum occurred at the second collection; it may have marked the end of the relatively rapid development of the orange toward maturity. As the season advanced the general level at which reducing sugar could be maintained (without being condensed to sucrose or other higher carbohydrates) became lower and lower, reaching a minimum during the hottest weather. This minimum perhaps marked a stage just previous to rapid deterioration. It was the time when the rind was freest from green color, and it coincided with the invert-sugar maximum. After this period there was an apparent reversal of the processes and the rinds began to "go back" in color, or become greener, particularly at the stem end. The reducing sugar increased and the invert sugar decreased, so that, as the temperatures dropped in autumn nearly to those of April or early May, the chemical picture within the Valencia rinds became similar in this respect to the one early in the season.

In the Washington Navel variety total sugar increased continually through the season, largely because of the steady increase in reducing sugar (fig. 2). Invert sugar fluctuated apparently more under the influence of immediate environmental factors. The graph of invert sugar is somewhat the inverse of the mean-temperature graphs, although there was apparently a very small increase as the season progressed.

*Nitrogen.*—There were no large seasonal changes in the percentage of total nitrogen, which was determined only in the Valencia variety. The individual portions of the rind showed numerous apparent fluctuations not only in the percentage of but also in the relations between soluble and insoluble nitrogen. Whether the total quantity of nitrogen actually changed is difficult to determine on account of the much greater variations of other substances. There must have been changes in the form, because, for example, the nitrogen released upon the decomposition of chlorophyll seemed to have remained in the rind; at least, the percentage of total nitrogen continued to increase gradually to the time of the marked decline of the rind in September (fig. 2). Near the end of the observation period the percentage of total nitrogen began to diminish although the rind became greener.

*Hesperidin.*—Hesperidin was determined only in rinds of the Washington Navel variety, in which it was found to diminish during the winter to a little more than one-half the original percentage (fig. 2).

*Hydrogen-ion concentration.*—Hydrogen-ion concentration likewise was determined only in the rind of the Washington Navel variety. It changed very little during the entire period of observation although there was a very small but steady decrease during this period (fig. 2).

## COMPARISON OF STEM AND BLOSSOM ENDS

To make a general comparison of stem and blossom ends of the rinds, all determinations belonging to each end were brought together and the average values calculated (table 2). Each average represents the albedo and flavedo for all collections, both at Pomona and at Corona. Table 2 shows a striking difference between stem and blossom ends of the rinds of both varieties, certain phases of which were reported upon by Klotz and Haas (5, 7). The following differences are shown in table 2 and figure 3: The rind of the stem end exceeded that of the blossom end in percentage of water, reducing sugar, total sugar, soluble solids, and soluble nitrogen;<sup>8</sup> the blossom-end rind exceeded the stem-end rind in invert sugar, insoluble nitrogen,<sup>8</sup> hydrolyzable polysaccharides, insoluble solids, crude fiber,<sup>8</sup> and flavedo-albedo ratio. In the Washington Navel rinds hesperidin and active acidity were likewise higher in the blossom end. Few exceptions to these differences occurred on any collection date or in either locality.

In addition, there were some very interesting seasonal variations in the difference between the two end rinds, particularly in the Valencia variety. The general summary as given in table 2 cannot disclose such seasonal trends; however, figure 3 shows how the difference between the stem and blossom ends fluctuated through the season. In Valencia oranges the difference in sugars tended to increase as the season advanced until the September 10 collection, when the maximum divergence was reached. If the graphs for sugars in Valencia oranges (fig. 3) are compared with the mean-temperature graph (fig. 1) it will be seen that the graphs are remarkably similar, indicating that the difference between the composition of the stem and blossom ends was a variable influenced by the temperature and that the higher the mean temperature the greater was the divergence. The graphs of figure 3 also show the seasonal divergence or difference between the two end rinds as regards insoluble solids, soluble solids, and hydrolyzable polysaccharides. In Valencia oranges these constituents do not exhibit a similar close parallelism with the mean-temperature graphs (as shown in figs. 1 and 2), but they agree in giving the greatest seasonal divergence for these substances as occurring at the September 10 collection. The seasonal-divergence graphs for water and nitrogens presented in figure 3 are similar to each other but do not parallel the mean-temperature graph. On analysis of the variations of the two rinds in respect to water it was found that in every case such variations were caused by the more rapid loss or the slower gain of water by the blossom end of the orange. This finding agrees with observations on the diurnal variations of water in Valencia rinds referred to on page 728, when it was noted that the blossom end showed somewhat the larger diurnal deficit.

Some emphasis has been placed here upon the seasonal variation of the difference between the two end rinds for the reason that if there is any significance in the many recorded differences between the ends of Valencia rinds in relation to storage and other spotting it should be of interest to know that the difference is strongly affected by seasonal factors.

<sup>8</sup> Determined only in the Valencia variety.

TABLE 2.—A comparison of the chemical composition of orange stem-end and blossom-end rinds<sup>1</sup>

VALENCIA								
Portion of rind	Reducing sugar		Invert sugar		Total sugar		Soluble solids	
	Average	Range	Average	Range	Average	Range	Average	Range
	<i>Percent</i>	<i>Percent</i>	<i>Percent</i>	<i>Percent</i>	<i>Percent</i>	<i>Percent</i>	<i>Percent</i>	<i>Percent</i>
Stem end.....	24.59	21.91-26.39	10.30	6.95-11.96	34.89	33.11-37.24	57.57	50.98-61.20
Blossom end.....	15.69	11.03-19.05	10.51	7.98-14.05	26.20	24.85-28.43	50.24	47.62-52.80
Portion of rind	Insoluble solids		Hydrolyzable polysaccharides		Soluble nitrogen		Insoluble nitrogen	
	Average	Range	Average	Range	Average	Range	Average	Range
	<i>Percent</i>	<i>Percent</i>	<i>Percent</i>	<i>Percent</i>	<i>Percent</i>	<i>Percent</i>	<i>Percent</i>	<i>Percent</i>
Stem end.....	32.56	28.12-36.25	10.17	7.20-12.69	0.439	0.386-0.517	0.432	0.397-0.464
Blossom end.....	39.05	36.69-42.97	14.53	11.43-17.73	.401	.366-.451	.496	.476-.530
Portion of rind	Total nitrogen		Water		Crude fiber		Flavedo-albedo ratio	
	Average	Range	Average	Range	Average	Range	Average	Range
	<i>Percent</i>	<i>Percent</i>	<i>Percent</i>	<i>Percent</i>	<i>Percent</i>	<i>Percent</i>	<i>Percent</i>	<i>Percent</i>
Stem end.....	0.871	0.801-0.958	76.9	74.9-79.5	22.39	20.11-27.47	1.54	1.36-1.67
Blossom end.....	.897	.853-.980	68.9	67.7-69.8	24.52	23.26-26.95	1.74	1.60-1.87
WASHINGTON NAVEL								
Portion of rind	Reducing sugar		Invert sugar		Total sugar		Soluble solids	
	Average	Range	Average	Range	Average	Range	Average	Range
	<i>Percent</i>	<i>Percent</i>	<i>Percent</i>	<i>Percent</i>	<i>Percent</i>	<i>Percent</i>	<i>Percent</i>	<i>Percent</i>
Stem end.....	32.97	25.81-37.59	5.89	4.23-6.97	38.86	31.34-43.99	62.11	57.07-65.73
Blossom end.....	28.01	22.24-32.27	6.06	5.29-6.93	34.06	28.12-38.24	55.25	46.92-60.18
Portion of rind	Insoluble solids		Hydrolyzable polysaccharides		Water		Flavedo-albedo ratio	
	Average	Range	Average	Range	Average	Range	Average	Range
	<i>Percent</i>	<i>Percent</i>	<i>Percent</i>	<i>Percent</i>	<i>Percent</i>	<i>Percent</i>	<i>Percent</i>	<i>Percent</i>
Stem end.....	37.89	34.27-42.93	12.18	10.88-13.95	75.8	73.4-78.4	0.94	0.87-1.02
Blossom end.....	44.75	39.82-51.06	15.02	12.97-16.75	71.7	70.2-73.2	1.15	1.10-1.22
Portion of rind	Hesperidin		Hydrogen-ion concentration					
	Average	Range	Average	range				
	<i>Percent</i>	<i>Percent</i>	<i>pH</i>	<i>pH</i>				
Stem end.....	2.59	0.56-3.96	5.82	5.48-6.08				
Blossom end.....	3.38	2.67-8.78	5.56	5.27-5.74				

<sup>1</sup> All values except water and hydrogen-ion concentration are calculated on the basis of dry weight.



## COMPARISON OF THE FLAVEDO AND ALBEDO

Table 3 gives a summary of all analyses arranged in such a manner as to allow comparison between the composition of the outer and the

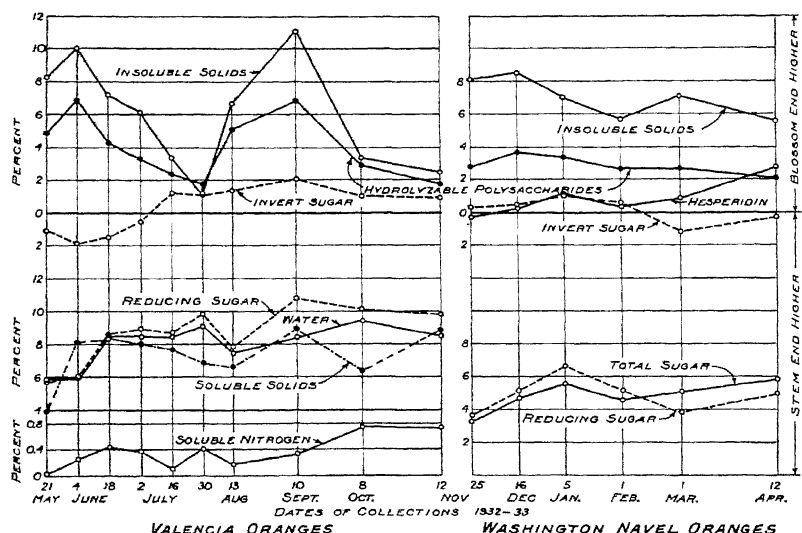


FIGURE 3.—Seasonal differences between stem and blossom ends with respect to certain constituents of the rind of Valencia and Washington Navel oranges.

inner layers of Valencia and Washington Navel orange rinds. The principal differences observed may be stated briefly as follows: In the Valencia rinds the flavedo always exceeded the albedo in weight (as indicated by the fact that the ratio always exceeded 1) and in nitrogen, but the albedo exceeded the flavedo in crude fiber. In both varieties the flavedo also had a higher percentage of reducing sugar, invert sugar, total sugar, and soluble solids; the albedo had a higher percentage of insoluble solids and hydrolyzable polysaccharides. There was no marked difference in the percentage of water. In the Washington Navel variety the albedo was higher in hesperidin and active acidity.

In Valencias the ratio of flavedo to albedo was always greater than unity. At the beginning of the Valencia season it was 1.57, increasing to a maximum of 1.77 at the September 10 collection. The variation of this ratio seemed to be due largely to changes within the albedo.

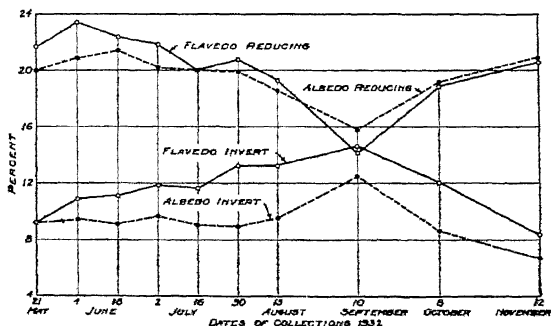


FIGURE 4.—Comparison of seasonal changes in the flavedo and albedo layer of the rind of Valencia oranges with respect to the sugar content.

TABLE 3.—A comparison of the chemical composition of orange flavedo and albedo<sup>1</sup>

## VALENCIA

Portion of rind	Reducing sugar		Invert sugar		Total sugar		Soluble solids	
	Average	Range	Average	Range	Average	Range	Average	Range
Flavedo.....	Percent 19.74	Percent 16.53-23.35	Percent 11.62	Percent 8.23-14.57	Percent 31.36	Percent 29.25-34.44	Percent 53.69	Percent 48.81-56.62
Albedo.....	19.01	16.16-21.76	9.12	6.69-11.44	28.13	27.87-30.92	51.44	48.22-54.21

Portion of rind	Insoluble solids		Hydrolyzable polysaccharides		Soluble nitrogen		Insoluble nitrogen	
	Average	Range	Average	Range	Average	Range	Average	Range
Flavedo.....	Percent 25.80	Percent 22.93-28.43	Percent 8.12	Percent 5.14-10.98	Percent 0.503	Percent 0.462-.591	Percent 0.525	Percent 0.496-0.533
Albedo.....	48.54	44.00-52.99	17.04	12.78-20.07	.328	.270-.384	.408	.358-.449

Portion of rind	Total nitrogen		Water		Crude fiber		Flavedo-albedo ratio	
	Average	Range	Average	Range	Average	Range	Average	Range
Flavedo.....	Percent 1.028	Percent 0.976-1.067	Percent 72.9	Percent 71.7-74.5	Percent 17.68	Percent 17.1-17.5	Percent 1.64	Percent 1.57-1.77
Albedo.....	.736	.665-.832	72.6	71.1-75.0	31.50	31.1-31.9	-----	-----

## WASHINGTON NAVEL

Portion of rind	Reducing sugar		Invert sugar		Total sugar		Soluble solids	
	Average	Range	Average	Range	Average	Range	Average	Range
Flavedo.....	Percent 32.85	Percent 26.40-37.34	Percent 7.17	Percent 5.52-7.87	Percent 40.02	Percent 32.37-44.79	Percent 65.51	Percent 58.96-69.71
Albedo.....	28.09	22.39-32.35	4.76	4.00-5.38	32.85	26.83-37.43	51.76	47.08-56.21

Portion of rind	Insoluble solids		Hydrolyzable polysaccharides		Water		Flavedo-albedo ratio	
	Average	Range	Average	Range	Average	Range	Average	Range
Flavedo.....	Percent 34.40	Percent 30.20-41.04	Percent 10.73	Percent 8.53-13.36	Percent 73.3	Percent 72.0-75.5	Percent 1.04	Percent 0.84-1.24
Albedo.....	48.24	43.79-52.97	16.46	15.02-17.77	74.3	72.2-76.3	-----	-----

Portion of rind	Hesperidin		Hydrogen-ion concentration	
	Average	Range	Average	Range
	Percent	Percent	pH	pH
Flavedo.....	2.42	2.02-3.08	6.10	5.60-6.38
Albedo.....	3.47	1.32-5.57	5.25	5.07-5.40

<sup>1</sup> All values except water and hydrogen-ion concentration are expressed on the basis of dry weight.

The seasonal trends of sugars in the flavedo and albedo of Valencia rinds are shown in figure 4. The graphs for reducing sugar and invert sugar show the characteristic behavior of these substances. The hot-weather period of August and early September diminished the reducing sugar and increased the invert sugar decidedly. The color of the fruit remained good until after the September 10 collection, when it began to regreen rapidly. The flavedo and albedo did not differ much in their reducing-sugar content, but the former was considerably higher in invert sugar throughout the season.

#### COMPARISON OF POMONA AND CORONA FRUIT

One of the interesting facts shown by the data was the very considerable difference in the chemical composition of the rind of Valencia oranges in the two localities, Pomona and Corona. Colby (2) published the results of a comparative study of sugars and acid in the juice of Washington Navel oranges from different sections of California, but the writers have seen no comparative observations on the rind.

A summary of the writers' results is presented in table 4. Each value given in the table is the average of 80 or more determinations.

In Valencias the rind of the Pomona fruit had a higher content of water, reducing sugar, soluble solids, soluble nitrogen, and total nitrogen. On the other hand, the rind of the Corona Valencias was higher in invert sugar, insoluble solids, hydrolyzable polysaccharides, and the flavedo-albedo ratio. Total sugar and insoluble nitrogen show very little significant difference.

The rind of Pomona Washington Navel oranges was higher in water, hesperidin, hydrolyzable polysaccharides, and insoluble solids, but lower in reducing sugar, invert sugar, total sugar, soluble solids, hydrogen-ion concentration, and flavedo-albedo ratio. The fruit at Corona, as already stated, was somewhat more advanced in maturity on all collection dates, and therefore the fruit from the two localities was not strictly comparable.

TABLE 4.—A comparison of the chemical composition of the rind of oranges grown in two localities during the season of 1932-33<sup>1</sup>

VALENCIA										
Locality	Reducing sugar	Invert sugar	Total sugar	Soluble solids	Insoluble solids	Hydrolyzable polysaccharides	Soluble nitrogen	Insoluble nitrogen	Total nitrogen	Water
	Pct.	Pct.	Pct.	Pct.	Pct.	Pct.	Pct.	Pct.	Pct.	Pct.
Pomona, Calif. ....	21.12	8.88	30.60	54.18	34.98	11.24	0.479	0.451	0.930	74.9
Corona, Calif. ....	17.89	11.67	29.56	50.00	40.07	13.78	.360	.480	.840	70.6
WASHINGTON NAVEL										
Locality	Reducing sugar	Invert sugar	Total sugar	Soluble solids	Insoluble solids	Hydrolyzable polysaccharides	Water	Flavedo-albedo ratio	Hesperidin	Hydrogen-ion concentration
	Pct.	Pct.	Pct.	Pct.	Pct.	Pct.	Pct.	Pct.	Pct.	Pct.
Pomona, Calif. ....	23.83	5.88	24.41	57.20	42.80	14.23	74.5	1.09	3.08	5.59
Corona, Calif. ....	22.15	6.35	28.50	60.06	39.94	13.06	78.0	1.09	2.28	5.35

<sup>1</sup> All values except water and hydrogen-ion concentration are calculated on the basis of dry weight.

Another factor that should be taken into consideration in this comparison is the rise in temperature that occurred at Corona during the interval between the pickings of Washington Navel at the two locations in December and January. The data were examined to find whether this difference in temperature for the few days preceding picking might have helped to widen the difference in composition of the rinds of fruit from the two localities. Graphs were constructed to show the seasonal divergence, with regard to certain substances, between the stem end and the blossom end (fig. 3) and between Pomona and Corona Washington Navel rinds taken as a whole (fig. 5). It was noted at once that the extent of divergence between the stem

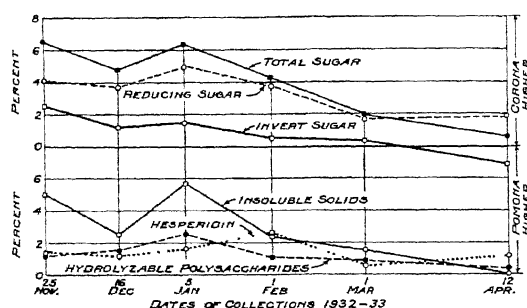


FIGURE 5.—Seasonal differences between Washington Navel oranges from Corona and Pomona with respect to certain constituents of the rind.

temperature changes at Corona on the dates stated may have affected the composition of the rinds to an extent measurable by the methods employed in this study.

#### CHANGES UNDER STORAGE CONDITIONS

##### VALENCIA ORANGES

During the November 12 collection extra quantities of fruit were picked and placed in cold storage in order to observe the changes that might take place in the rinds.

The temperatures used were 33° and 53° F. A controlled, high relative humidity was maintained in the 53° room but no control of relative humidity in the 33° room. In the latter the relative humidity was always lower than in the former; in fact, enough lower to more than offset the lower vapor tension at 33°, with the result that the rinds of this lot lost noticeably more water. The period of storage was 7 weeks (Nov. 12 to Dec. 31). When the final examination was made the fruit seemed in fair condition, except for the characteristically "old" appearance and considerable shrinkage in the lot held at 33°. The difference in rate of water loss in the two storage rooms renders the interpretation of the chemical data difficult. It is clear that all such experimental storage rooms ought to be maintained at equivalent relative humidities so that the loss of water from the fruit would be approximately the same in every room.

The stem-end rinds lost water faster than the blossom-end rinds. Apparently there can be a significant difference in the behavior of the two ends in this regard, depending upon whether or not the fruit is attached to the tree. The flavedo-albedo ratio varied in storage;

the flavedo nearly always lost water more rapidly than the albedo. It is not known whether this was due to the greater exposure of the flavedo or to its smaller water-holding capacity. Some tests were made of the rate of drying when flavedo and albedo tissues were exposed in the laboratory. The flavedo lost about 26 percent of its original weight in 4½ hours, whereas the albedo lost 16 percent. Weighings at the end of 24 hours showed that the albedo tissue had continued to lose water at nearly a constant rate, making a total of 62 percent, but the rate of drying of the flavedo had so decreased after the first period that the total loss was only 44 percent. It would be interesting to test the successive changes in flavedo-albedo ratios of oranges during storage at low humidity. Soluble solids decreased in all portions of the rinds at both temperatures, and the decrease was more than twice as much at 53° as at 33° F. When the stem end and the blossom end were compared it was found that the stem-end rind had lost twice as much of its soluble solids as the blossom end.

A diminution of total sugars accounts for a large part of the change in soluble solids. Reducing sugar decreased slightly, but invert sugar decreased greatly. The latter almost disappeared when the fruit was held at 53° F. There was an apparent small increase of hydrolyzable polysaccharides in all portions of the rind in both storage rooms. However, it is doubtful whether this is a real increase, for it is decidedly contrary to the results obtained for polysaccharides during extensive companion studies of the rinds of grapefruit in storage. The changes in soluble and insoluble nitrogen in storage were so slight that no correlations were noted.

The results obtained in the determination of the hydrogen-ion concentration in the rind and in the pulp juice of the two storage lots from Pomona are presented in table 5. From these data it is evident that the rind of the fruit from the 33° F. room was more acid than that from the 53° room. This is true of all corresponding portions of the rind, the average difference being 0.26 pH. The stem-end portions of the rind were more acid than those from the blossom end, and the equatorial portions held an intermediate position. The flavedo was less acid than the albedo. The pulps showed a difference of only 0.04 pH, but in this case that held at 53° was more acid.

TABLE 5.—*Hydrogen-ion concentration determinations of different portions of Valencia oranges after being held in storage for 7 weeks at two different temperatures*<sup>1</sup>

Storage temperature (°F.)	Stem-end rind	Blossom-end rind	Equatorial rind	Flavedo	Albedo	Average of rind portions	Pulp juice
	pH	pH	pH	pH	pH	pH	pH
53	5.97	6.33	6.23	6.60	5.77	6.18	3.78
33	5.79	6.01	5.96	6.32	5.51	5.93	3.82
Difference	.18	.32	.27	.28	.26	.26	.04

<sup>1</sup> Each pH value is the average of 16 determinations. The writers are fully aware that the averaging of pH values, here and elsewhere in this paper, is mathematically unsound, but they believe that through the ranges shown, the averages of pH values as numerical entities are probably as sound biologically as the averages of the actual hydrogen-ion concentrations or the equivalent pH values, and are considerably more convenient.

#### WASHINGTON NAVAL ORANGES

The first storage experiment of this study was started at a time corresponding to the third field collection. The fruit came from the same trees at Pomona and Corona that were used for the regular field

collections. The fruit from each of the two localities was divided into two lots and placed in storage on January 7 and 11, respectively. The length of the storage period was 30 days. One lot from each locality was held at 52° F. with an average relative humidity of 89 percent, and the other lot was held at 32° with an average relative humidity of 76 percent. At the end of this period the fruit was free from rind blemishes at both temperatures. Chemical analyses of the rinds of these and the following lots were carried through exactly as described for the regular field collections.

The time of collection for the second storage experiment corresponded with that of the fifth field collection, March 1 to 4. Lots from each locality were stored for 46 days at 52°, 42°, and 32° F. with average relative humidities of 89 percent (range 85-93 percent), 85 percent (range 83-86 percent), and 85 percent (range 83-87 percent), respectively. At the end of this time none of the fruit showed any rind blemishes except the lot from Corona held at 32°. In this lot 72 percent of the fruit showed a brown stain somewhat resembling common apple scald.

The third storage experiment was carried out with Corona fruit only, and the time of picking corresponded with that of the sixth field collection, April 13. The temperatures and relative humidities used were the same as for the second storage experiment. The length of the storage period was 33 days, after which the rinds were found to be free from storage blemishes except in the lot held at 32° F. All of this fruit showed various amounts of brown stain.

The fourth storage experiment was made with Pomona fruit only, and the time of picking corresponded with that of the seventh<sup>9</sup> field collection, May 25. The temperatures and relative humidities were again the same as those used in the second storage experiment. The length of storage was 41 days. At the end of this period there were no storage blemishes on the rinds except in fruit of the lot held at 32° F., all of which showed brown stain. The fruit had been picked after the regular commercial season, and none of it was very firm when placed in storage; at the time of removal from storage it was somewhat soft and not fresh looking. This last statement applies nearly as well to the Corona fruit used in the third storage experiment. This fruit had been picked nearly 6 weeks before the Pomona fruit of the fourth storage experiment, yet the fruit of both locations seemed to be at about the same stage of maturity when placed in storage.

#### EFFECTS OF TEMPERATURE

##### ON RIND AS A WHOLE

External observations of the oranges used in the simple storage experiments described above pointed to two tentative conclusions, namely, (1) the fruit was most susceptible to brown stain at 32° F., and (2) it was also more susceptible during the latter part of the season.

The effects of storage temperatures on the chemical composition of Washington Navel orange rinds are shown in table 6. The values in this table are from the data from the second, third, and fourth storage experiments. Data obtained from the first storage experiment are not included because there was no storage at 42° F.

<sup>9</sup> Since, as already stated, the 7th field collection was made only at Pomona, to serve as a control for the storage experiment, the analytical data from it were not included in the presentation of changes in the field but were used for the average percentages at beginning of storage given in table 5.

It is evident from table 6 that certain substances in the rind gave definite responses to the temperatures used. Invert sugar showed only a small decrease at 32° F. but a very large one at 52°. Reducing sugar showed a smaller decrease, which was progressively reduced as the temperature of storage was lowered. The striking alteration in invert sugar had little effect on the general trend of total sugar because invert sugar comprised scarcely more than one-tenth the amount of reducing sugar. Total sugar, therefore, behaved much like reducing sugar.

TABLE 6.—*Effects of temperature on the composition of the rind of the Washington Navel orange in storage*<sup>1</sup>

Storage conditions	Reducing sugar		Invert sugar		Total sugar		Soluble solids	
	Con- tent	Loss	Con- tent	Loss	Con- tent	Loss <sup>2</sup>	Con- tent	Loss
Before storage.....	Percent 34.67	Percent	Percent 5.94	Percent	Percent 40.61	Percent	Percent 63.0	Percent
After 46 days at—								
52° F.....	33.43	3.58	3.14	47.1	36.57	9.9	60.4	4.2
42° F.....	34.01	1.91	3.66	38.4	37.67	7.2	60.7	3.7
32° F.....	34.25	1.21	5.46	8.1	39.71	2.2	62.8	.3

Storage conditions	Insoluble solids		Hydrolyzable polysaccharides		Hesperidin		Hydrogen-ion concentration	
	Con- tent	Gain	Con- tent	Loss (-) or gain(+)	Con- tent	Gain	pH	Differ- ence in pH
Before storage.....	Percent 37.0	Percent	Percent 11.89	Percent	Percent 2.12	Percent	5.94	
After 46 days at—								
52° F.....	39.6	7.1	12.57	+5.72	3.14	48.1	5.47	0.47
42° F.....	39.3	6.2	12.41	+2.29	3.04	43.4	5.52	.42
32° F.....	37.2	.54	11.79	-.93	2.65	25.0	5.83	.11

<sup>1</sup> All percentages are calculated on the basis of dry weight.

<sup>2</sup> The percentage of loss or gain is calculated on the basis of the percentage present at the beginning of the storage period.

The recorded increase of hesperidin in storage is very interesting. At 52° F. hesperidin increased to one and one-half times the quantity present at the beginning of storage, and even at 32° it increased to one and a quarter times that originally present. That this is an actual increase in the quantity of hesperidin seems certain, for if the loss of sugar and other solids is taken into account, there is still a clear margin showing increase of this substance at all temperatures used. This synthesis of hesperidin in storage seems to be correlated with the rate of respiration, and its accumulation with the lack of translocation. Hesperidin was found to decrease steadily through the season while the fruit was on the tree. This fact suggests a number of possibilities, of which one is that while translocation of this rather stable glucoside (or group of glucosides) can continue while the fruit is on the tree, there may be a diminution of the rate of respiration as the orange matures. Thus one might account for the seasonal decrease. It can scarcely be assumed that the synthesis of hesperidin ceases completely at any time in the field.

Soluble solids showed the greatest loss at 52° F. and the least at 32°. The insoluble solids consequently had an apparently opposite trend.

Hydrolyzable polysaccharides apparently increased in storage at 52° and 42° F., but if the loss of other substances is taken into account it is found that they remained practically stationary, or possibly decreased slightly at all temperatures.

When the rinds were considered as a whole it was found that the flavedo-albedo ratio remained practically unchanged in storage. However, if stem and blossom ends were compared separately it was found that in the stem end the ratio made an average increase of 0.04 at 32° F., a decrease of 0.03 at 42°, and a decrease of 0.04 at 52°. In the blossom end the changes were almost exactly reversed, the changes in value of the ratios being a decrease of 0.04 at 32°, a decrease of 0.03 at 42°, and an increase of 0.06 at 52°.

Under all conditions of storage there was an increase of active acidity in all portions of the Washington Navel orange rind (table 7). The greatest increase was at 52° F. and the least at 32°. This effect of temperature is quite the reverse of that recorded for the Valencia orange rind, which will be referred to again in the comparison of the two orange varieties.

#### ON PORTIONS OF RIND

In all the preceding discussion of the effects of different temperatures on the rind of Washington Navel oranges in storage, the rind has been considered as a whole, except for a brief mention of the flavedo-albedo ratios in the rind of the stem and blossom ends.

A detailed account of the relative behavior of the stem and blossom ends, of the flavedo and albedo, or of fruit from the two localities, would seem undesirable in a summarized report of this kind. Nevertheless, it may be stated that the different portions of the rind did often respond to storage conditions somewhat differently, although these responses differed in quantity only.

TABLE 7.—*Hydrogen-ion concentration of different portions of the rind of Washington Navel oranges held in storage 46 days at different temperatures*

Storage temperature (° F )	Stem end	Blossom end	Flavedo	Albedo	Average <sup>1</sup>
	<i>pH</i>	<i>pH</i>	<i>pH</i>	<i>pH</i>	<i>pH</i>
Before storage (control) .....	8.15	5.74	6.39	5.49	5.94
52° .....	5.69	5.25	5.74	5.20	5.47
42° .....	5.75	5.29	5.79	5.24	5.52
32° .....	6.03	5.63	6.24	5.42	5.83
Average <sup>1</sup> .....	5.82	5.39	5.92	5.29	5.61

<sup>1</sup> See footnote 3 to table 1.

The stem end showed greater changes than the blossom end in all the substances under observation except active acidity. The immediate explanation of this was found to involve the relative distribution of the albedo tissue, which is more responsive to everything except acidity. The albedo is more abundant in the stem end (the flavedo-albedo ratio was less than unity here), and the reverse is true of the blossom end. Why the flavedo tissue of the Washington Navel



orange in storage should show greater change in active acidity than the albedo is not clear, but the condition appears to be quite different from that found in studies on the rind of grapefruit, which will be reported separately, where the albedo showed greater responses in acidity. This greater activity in acidity responses of the flavedo of the Washington Navel orange rind might possibly have some relation to the surface brown stains noted in storage, in contrast to the deeper seated pittings noted in the grapefruit rind where the albedo shows the greater acidity response.

#### COMPARISON OF RINDS OF WASHINGTON NAVAL AND VALENCIA ORANGES

When any attempt is made to compare the rinds of the Washington Navel and Valencia oranges in southern California, it must be kept in mind that almost none of the chemical differences between them can be attributed definitely to the fact that the oranges are of different varieties. It has already been mentioned that the two varieties are harvested at different seasons of the year. In southern California the Washington Navel orange season begins in the late fall and ends in the spring, whereas the Valencia orange season begins in the spring and extends through the summer. The average weather conditions affecting the two varieties at harvest time differ as widely as is possible for two intervals of such length in the district; for example, the mean temperature for the entire period during which the Washington Navel orange material was collected was 52.1° F., whereas the corresponding mean for the period of Valencia collections was 67.3°. During the season of the Washington Navel orange there were many minima of 32° or below, and in the Valencia season there were several maxima of 100° or above. The opinion has been advanced that the temperature and other environmental conditions immediately preceding the picking of the fruit are capable of producing considerable and rapid chemical changes within the rind tissue. If this be true it seems probable that recorded differences between Washington Navel and Valencia orange rinds will have been influenced by the seasonal differences in the weather affecting each variety immediately preceding and during picking. In other words, if the environmental conditions present at the harvest time of the two varieties were reversed, then the chemical situation in the rinds might also be reversed, or at least considerably altered. It is to be expected that such wide differences in temperature and other climatic factors at the time of harvest might induce a difference in the rinds of the two varieties that would be reflected in their behavior in storage and transit. With the foregoing caution against accepting differences between rinds of the Washington Navel and Valencia oranges as altogether varietal, the following comparisons are presented.

#### IN THE FIELD

A general summarized comparison, presented in table 8, shows the average of all chemical determinations for both varieties as they came from the grove. Each value for the Washington Navel rind in the table is the average of 96 determinations, and each value for the Valencia rind is the average of 160 determinations.

TABLE 8.—*A comparison of the chemical composition of the rind of Washington Navel and Valencia oranges before storage*<sup>1</sup>

Variety	Water	Reducing sugar	Invert sugar	Total sugar	Soluble solids	Hydrolyzable polysaccharides	Reducing-invert sugar ratio	Flavido-albedo ratio
	Percent	Percent	Percent	Percent	Percent	Percent	Percent	Percent
Washington Navel <sup>1</sup> .....	73.8	30.49	5.97	36.46	58.68	13.60	5.11	1.04
Valencia <sup>2</sup> .....	72.0	19.38	10.37	29.75	53.91	12.58	1.87	1.64

<sup>1</sup> All values except those in the first and the last 2 columns are calculated on the basis of dry weight.<sup>2</sup> Average of 96 determinations.<sup>3</sup> Average of 160 determinations.

The Washington Navel rind showed a slightly higher water content than the Valencia rind. This might easily be the effect of the higher temperatures and greater evaporating power of the air during the Valencia season. As to seasonal trend, the Washington Navel rind showed a small decrease and the Valencia rind a small increase as the season advanced.

Reducing sugar was more than one and one-half times as abundant in the Washington Navel rind. On the other hand, invert sugar was nearly twice as high in the Valencia rind. Total sugar, however, in the Washington Navel rind was nearly one-fourth more than that in the Valencia rind. The difference in value of the reducing-invert sugar ratio was surprising. In the Washington Navel rind this ratio was 2.7 times as great as in the Valencia rind.

During the season total sugars remained practically constant in the Valencia rind but increased steadily in the Washington Navel rind. The graph (fig. 2) showing seasonal variations in reducing sugar for the Valencia rind paralleled the temperature graph; in the Washington Navel rind reducing sugar increased steadily. The seasonal invert-sugar graph for Valencias was in general the inverse of the reducing sugar graph, but in the Washington Navel rind this was not true.

Soluble solids and hydrolyzable polysaccharides were more abundant in the Washington Navel rind. Soluble solids increased steadily throughout the season in this variety but increased or diminished almost inversely with the temperature in the Valencia rind.

Hydrogen-ion concentration was higher in the stem-end than in the blossom-end rind of Valencias, but in the Washington Navel rind the hydrogen-ion concentration was lower in the stem end. The hydrogen-ion concentration of the rinds as a whole was higher in the Washington Navel orange.

The average flavido-albedo ratios of Washington Navel and Valencia orange rinds were 1.04 and 1.64, respectively. The smaller ratio for the Washington Navel was due immediately to its considerably heavier albedo. The flavido also was somewhat heavier than that of the Valencia orange, but this difference was more than offset by the relatively heavier albedo.

It was noted that at Pomona the Valencia rind had more reducing sugar and soluble solids and less hydrolyzable polysaccharides than at Corona. In the Washington Navel rind conditions were exactly the reverse.

## IN STORAGE

For a comparison of the relative behavior of the Washington Navel and Valencia orange rinds in storage, the data from only two temperatures, 52° and 32° F., are available, since no Valencia oranges were stored at 42°.

Table 9 gives some average changes in the composition of the rinds of the two varieties in storage. In general, the Valencia rind made greater responses to storage conditions than did the Washington Navel orange rinds. It seems possible that the condition of the Valencias in the field, influenced as they were by hot, dry weather, might have brought about the greater changes that occurred in this variety in storage. For most collections, the temperature of the Washington Navel oranges was actually raised when taken from the field to storage at 52° F. Changes at this temperature were greater in both varieties, except in the hydrogen-ion concentration. In the Valencia rind the apparent increase of reducing sugar at 52° was probably due to the very abundant invert sugar, which for a time was hydrolyzed at a rate exceeding that at which it was consumed by respiration.

TABLE 9.—*Comparison of the composition of Washington Navel and Valencia orange rinds before and after storage at different temperatures*<sup>1</sup>

Variety and storage conditions	Reducing sugar	Invert sugar	Total sugar	Hydrolyzable polysaccharides	Soluble solids
	Percent	Percent	Percent	Percent	Percent
Washington Navel:					
Before storage.....	34.67	5.94	40.61	11.89	63.05
After 42 days at—					
52° F.....	33.43	3.14	36.57	12.57	60.44
32° F.....	34.25	5.46	39.71	11.79	62.78
Valencia:					
Before storage.....	21.09	7.47	29.16	10.12	61.20
After 48 days at—					
52° F.....	22.33	2.54	24.87	13.77	54.85
32° F.....	19.75	6.38	26.13	12.20	52.63

<sup>1</sup> All values are calculated on the basis of dry weight.

As mentioned in the presentation of the storage data for the Washington Navel orange rind, the changes in hydrogen-ion concentration in storage were strikingly different in the two varieties. The Valencia rind developed a greater acidity at 33° than at 53° F., the pH averages of which were 5.92 and 6.18, respectively, but the Washington Navel rind consistently showed the reverse effect, the average pH values being 5.83 and 5.47 at 32° and 52°, respectively.

## DISCUSSION

While it is freely admitted that the results reported cannot at present be definitely linked with pitting and spotting in storage, there are some general aspects of the findings that are worthy of notice.

Since orange rinds were found to respond sometimes very strikingly to changes in the environment, it is suggested that these responses probably relate closely to the behavior of the rinds after picking. Even a moderate understanding of this relationship might modify picking practices considerably, especially for fruit destined for

export or storage. At present the more vigilant packing-house managers do not wish Valencias picked during periods of unusually hot weather and insist that they shall not be picked except during a few hours in the early morning. So meteorology may come to play as important a role in the picking schedule as it now does in spraying and frost control.

It has been generally assumed that the susceptibility to spotting of fruit coming from the grove depends somewhat, at least, upon the district in which the fruit is grown, the season, stage of maturity, and general cultural treatment received in the grove. However, some of the responses of rinds observed in this work seem to indicate that a given orange on different days may present a significantly different aspect as regards its possible resistance to subsequent conditions in storage and transportation that favor pitting and spotting; that is to say, the susceptibility of an orange to spotting in storage is probably not merely "high" in very early and very late stages of maturity, but it may vary from week to week, being sometimes high and sometimes low, depending upon the environmental factors of the few days preceding the picking of the fruit.

A study of the relation of storage and transit conditions to pitting and spotting is still in progress.

#### SUMMARY AND CONCLUSIONS

Results are presented of a study of changes taking place in the rind of oranges in the field and in storage during the season of 1932-33 at Pomona and Corona, Calif.

Seasonal changes in the composition of the rind as a whole were as follows:

(1) In Valencia oranges there was a slight seasonal upward trend in water content, but in Washington Navels the reverse was true, while in both varieties there were fluctuations due to immediate environmental causes.

(2) In Valencias the flavedo-albedo ratio was always greater than unity, increasing temporarily in hot weather; in Washington Navels the ratio varied on either side of unity and likewise fluctuated with weather conditions.

(3) In Valencias the soluble solids varied inversely with the mean temperature, but in Washington Navels there was a general relative increase throughout the season.

(4) In Valencias there was practically no change in total sugar during the season but invert sugar increased as reducing sugar decreased. In Washington Navels, on the other hand, total sugar increased throughout the season, primarily because of the steady increase of reducing sugar; invert sugar fluctuated with immediate environmental factors.

(5) In Valencias the hydrolyzable polysaccharides varied with the mean temperature, but in Washington Navels they decreased as the season advanced.

(6) Nitrogen content was studied only in Valencia oranges. Only a slight change was observed during the season, with some fluctuations in the proportion of the soluble and insoluble fractions.

(7) Hesperidin and hydrogen-ion concentration were determined only in the rinds of Washington Navel oranges. Hesperidin decreased

steadily during the season to about one-half the amount present early in the season. Hydrogen-ion concentration made a small but definite decrease during the season.

A comparison of the stem and blossom ends showed a definite difference in composition during the season. In Valencias the stem end was higher in water, reducing sugar, soluble nitrogen, and soluble solids, while the blossom end had a greater amount of total sugar per 100 ml of solute than the stem end because of the higher water content of the stem end. These differences varied through the season, being greatest during the hottest weather. In Washington Navels the stem end was likewise higher in water, reducing sugar, total sugar, and soluble solids and the blossom end was higher in invert sugar, hydrolyzable polysaccharides, insoluble solids, hesperidin, hydrogen-ion concentration, and flavedo-albedo ratio.

A comparison of the flavedo and albedo showed that in the Valencia the flavedo always weighed more than the albedo and that while there was some variation in the ratio in the Washington Navel it averaged more than unity. In the Valencia the flavedo contained a higher percentage of invert sugar, total sugar, soluble solids, and nitrogen. In the Washington Navel the flavedo was likewise higher in percentage of reducing sugar, invert sugar, and soluble solids. The albedo was higher in water, insoluble solids, and hydrolyzable polysaccharides. In the Washington Navel the albedo was higher in hesperidin and hydrogen-ion concentration. In Valencia rinds the flavedo-albedo ratio was highest during the hottest weather.

When oranges from Pomona were compared with those from Corona the rinds were found to differ in several respects. In Valencias the Pomona fruit was higher in water, reducing sugar, soluble solids, and total nitrogen, while in the Washington Navel it was higher in water, hesperidin, hydrolyzable polysaccharides, and insoluble solids. The Corona Valencias were higher in invert sugar, insoluble solids, hydrolyzable polysaccharides, and flavedo-albedo ratio, while the Corona Washington Navels were higher in both reducing and invert sugars, soluble solids, hydrogen-ion concentration, and flavedo-albedo ratio.

Storage experiments were conducted to determine the response at different temperatures. With Valencias storage experiments at 33° and 53° F. for 7 weeks gave the following results: (1) The stem-end rind lost water more rapidly than did the blossom-end rind; (2) soluble solids always decreased more rapidly at 53° than at 33°; (3) the stem-end rind lost soluble solids twice as rapidly as did the blossom-end rind; (4) reducing sugar changed very little and invert sugar almost disappeared at 53°; (5) rind from fruit stored at 33° had a higher hydrogen-ion concentration than that stored at 53°; and (6) the stem-end rind of stored fruit had a higher hydrogen-ion concentration than did the blossom-end rind. There was practically no difference in the active acidity of the pulp juice of the two lots.

In the storage experiments on Washington Navel oranges the temperatures employed were 52°, 42°, and 32° F. The responses to storage conditions were as follows: (1) The stem-end rind always showed greater responses to storage conditions than the blossom-end rind except in acidity; (2) in all portions of the rind and under all storage conditions acidity increased, the increase being greatest at 52° and least at 32°; (3) invert sugar decreased slightly at 32° but

greatly at 42° and 52°; (4) reducing sugar changed much less than invert sugar but in the same direction; (5) hesperidin showed very definite increases in storage, the increase being relatively greatest at 52°; (6) soluble solids decreased most at 52°; (7) hydrolyzable polysaccharides changed very slightly at any temperature; (8) the flavedo-albedo ratio remained unchanged in the rind as a whole, but there was a small increase of the ratio in the stem end that was exactly offset by a decrease in the blossom end.

In considering the results on Washington Navel and Valencia oranges it must be remembered that in southern California the Washington Navel oranges are harvested in winter and the Valencias in summer, which makes it difficult, in a comparison of the two varieties as grown there, to distinguish between the differences which are varietal and those which are caused by environment.

The results of storage experiments with Washington Navel oranges indicate that late-picked fruit is most susceptible to brown stain and that susceptibility is increased by storage at 32° as compared with 42° and 52° F. With respect to pitting and spotting the results are not so clear-cut but seem to show that susceptibility to these disorders varies with the environmental factors of the last few days preceding the picking of the fruit.

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# FIELD AND STORAGE STUDIES ON CHANGES IN THE COMPOSITION OF THE RIND OF THE MARSH GRAPEFRUIT IN CALIFORNIA<sup>1</sup>

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## INTRODUCTION

This paper presents the results of two closely related studies. The first section deals with the rind of the Marsh grapefruit (*Citrus grandis* (L.) Osbeck) in the field during the harvest season, and the second section deals with the rind of comparable fruit held in storage at different temperatures or under other experimental conditions.

As in the major portion of the studies on Valencia and Washington Navel orange rinds previously reported (13),<sup>2</sup> the object of the first study was to determine, as closely as the methods employed would permit, the normal seasonal changes of grapefruit rind in the field. The experiments were so designed that the information obtained might be used as the basis for a rather extensive investigation of the behavior of grapefruit rind in storage. The results of this latter study are reported in the second part of this paper.

## REVIEW OF LITERATURE

The literature of previous work on the grapefruit rind is similar to that noted in connection with the orange (13) rind in that the attention of investigators has been directed mainly to the essential oils from a pharmaceutical or related standpoint, and when other substances have been taken into account the normal seasonal trends have not usually been considered.

One of the most extensive series of observations on seasonal changes in grapefruit is that reported by Lopez-Dominguez (17, 18) dealing with Duncan and Marsh varieties. He noted changes in composition of the fruit as affected by maturity, soil type, and rainfall. The more significant portion of his report concerns the changes in the pulp and juice, but information is also given on seasonal variations in thickness of the rind. In both varieties the rind decreased in thickness with maturity; in the Marsh the rate of decrease was checked when the solids-acid ratio reached 7. Hawkins (14) in his study of grapefruit ripening noted that the percentage of rind decreased throughout the season and that this was paralleled by a decrease in thickness. The changes taking place in the pectic constituents of the albedo of Florida grapefruit rind have been studied by Gaddum (8). Caldwell (2) has followed the changes in hydrogen-ion concentration and chemical composition in entire Florida grapefruit, beginning with extremely immature stages and including samples taken at weekly intervals until the beginning of the commercial harvest period.

<sup>1</sup> Received for publication Aug. 30, 1935; issued June 1936.

<sup>2</sup> Reference is made by number (italic) to Literature Cited, p. 786.

As regards seasonal development of the fruit his study ends about where the present one begins, and does not deal at all with the rind alone.

Other reports dealing with the analysis of the grapefruit rind do not refer to successive changes but present observations either on the distribution of certain substances (other than essential oils) in the rind or on the differences in composition between different portions of the rind. For example, Willimott and Wokes (22, 23) found that in grapefruit peroxidases are concentrated in the flavedo, although these substances are fairly widely distributed in the rinds of orange and lemon. These authors also studied the distribution of vitamins in grapefruit rind. Klotz and Haas (16) observed that the stem end of grapefruit had a greater permeability than the blossom end.

## FIELD STUDIES

### MATERIALS AND METHODS

Two field studies were made. One was carried out in the district that includes Fontana and Corona, Calif.; and the other was carried out in a single locality near Oasis, Calif., in the Coachella Valley.

The field portion of the first study extended through 171 days, beginning March 19 and ending September 6, 1933. This spring and summer period coextends in general with the regular harvest of grapefruit in the western part of southern California.

The second study began November 6, 1933, and ended April 9, 1934, a period of 154 days. This winter and spring period also coextends in general with the commercial harvest of grapefruit in the Imperial and Coachella Valleys.

These observations allow a comparison of rinds of the same variety of grapefruit grown in two districts so distinctly different in climate that in one the harvest time is in the summer and in the other in the winter. This difference in harvest seasons is practically the same as that encountered in regard to the Valencia and Washington Navel oranges (13), but in the latter case the two varieties were grown in the same climatic district.

The mean temperatures at Fontana or Corona and at Oasis during the periods of observation differed less than might have been expected, but the climatic environment differed greatly. In the Coachella Valley the harvest season follows an extremely hot and dry summer, while in the Fontana-Corona district it follows a relatively cool period.

All fruit used in this study was obtained from trees carefully selected at the beginning of the season, five each at Fontana and Corona, and six at Oasis. The soil types in which the experimental trees were growing at Fontana, Corona, and Oasis were Hanford gravelly sand, Yolo gravelly loam, and Superstition sand, respectively.

Collections were made at intervals of approximately 6 weeks. At Corona and Fontana 10 fruits were picked from each of the 5 selected trees and at Oasis 8 fruits were picked from each of the 6 trees. The fruit was brought into the laboratory, where the rinds were removed and separated into four portions as follows: (1) Stem-end flavedo, (2) stem-end albedo, (3) blossom-end flavedo, and (4) blossom-end albedo.



The procedure followed in the determination of hydrogen-ion concentration was the same as that described in the report on Washington Navel orange rind (13), except that when this determination was made in the pulp juice the tissue was not frozen before pressing and the juice was diluted to four times its original volume before the analysis. The samples of pulp juice were also titrated for total acidity. The titrations were made in duplicate, using 50 ml of juice for each and diluting with 100 ml of distilled water; normal sodium hydroxide was used for the neutralization, with phenolphthalein as indicator.

Naringin was determined by the method used for hesperidin in the Washington Navel orange rind (13), except that the weight of the derivative sugar was multiplied by the factor 1.60 instead of 2.17 in order to obtain the weight of anhydrous naringin. The procedures in all other analyses were the same as those described in the report on orange rinds (13).

The sugars were calculated as follows: (1) "Reducing sugar", as glucose, from the copper equivalent to the permanganate titration before inversion; (2) "invert sugar", as invert sugar, from the copper equivalent to the permanganate titration after inversion minus the permanganate titration before inversion; and (3) "total sugar", as the sum of "reducing sugar" plus "invert sugar."

Since the Fontana and Corona locations are so similar climatically, the data from them are handled together in all comparisons with the data from Oasis.

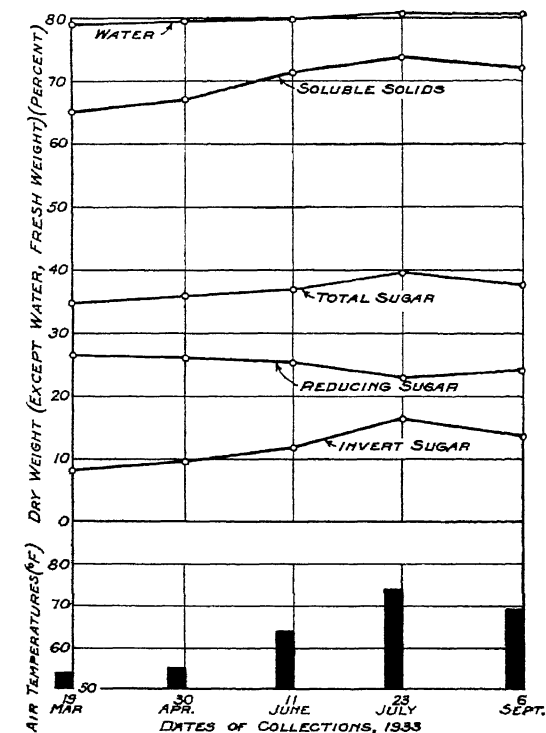


FIGURE 1.—Seasonal changes in mean air temperature (means for 5-day periods preceding collections) and in the composition of the rind of Marsh grapefruit from Corona and Fontana, Calif., with respect to water, soluble solids, and reducing, invert, and total sugars.

#### EXPERIMENTAL RESULTS

##### SEASONAL CHANGES

##### AT FONTANA AND CORONA

The seasonal changes in the composition of the grapefruit rind at Fontana and Corona are summarized in table 1 and figures 1 and 2. Each percentage value given in this table is the average of 16 determinations.

The water content of the rind did not change greatly, although there seemed to be a very slight upward trend through the season. This must mean that the water-holding power of the rind tissue increased also, otherwise there would have been a loss of water owing

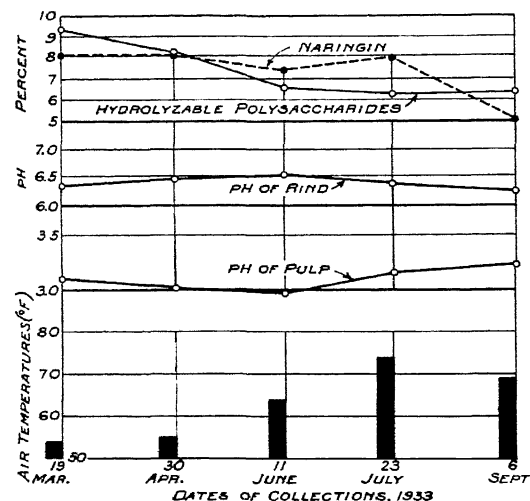


FIGURE 2.—Seasonal changes in mean air temperature (means for 5-day periods preceding collections) and in the composition of the rind of Marsh grapefruit from Corona and Fontana, Calif., with respect to naringin, hydrolyzable polysaccharides, and hydrogen-ion concentration. The pH of the pulp is also shown

to the fact that the season was growing warmer and less humid until the time of the fourth collection (July 23).

Reducing sugar decreased to a minimum at the fourth collection, whereas invert sugar increased to a maximum at that time (fig. 1). If the graphs representing the seasonal changes of these sugars are compared with the block graphs showing the mean air temperatures at Fontana and Corona, it will be noted that the reducing sugar changed somewhat inversely to and the invert sugar parallel to the temperature changes.

Total sugar behaved as did invert sugar, which dominated the total, not in percentage but by its greater seasonal changes.

TABLE 1.—Seasonal changes in the chemical composition of the rind of Marsh grapefruit grown at Corona and Fontana and at Oasis, Calif., during the season of 1933-34<sup>1</sup>

CORONA AND FONTANA, AVERAGES OF 16 DETERMINATIONS

Date of collection	Water	Reducing sugar	Invert sugar	Total sugar	Naringin	Hydrolyzable polysaccharides	Soluble solids	Insoluble solids	Hydrogen-ion concentration of rind	Flavado-albedo ratio	Hydrogen-ion concentration of pulp juice	Milli-liter N/1 acid in 10 ml pulp juice
	Percent	Percent	Percent	Percent	Percent	Percent	Percent	Percent	pH	Percent	pH	
1933												
Mar. 19	78.9	26.55	8.10	34.65	8.07	9.40	65.1	34.9	6.31	0.74	3.09	3.74
Apr. 30	79.4	26.09	9.53	35.62	8.07	8.33	67.1	32.9	6.45	.75	3.01	3.66
June 11	79.7	25.12	11.67	36.79	7.40	6.60	71.1	28.9	6.53	.77	2.97	3.47
July 23	80.7	28.06	16.30	39.36	7.97	6.23	73.7	26.3	6.36	.78	3.16	2.96
Sept. 6	80.6	23.81	13.63	37.44	6.07	6.40	72.2	27.8	6.24	.71	3.22	2.62

OASIS, AVERAGES OF 8 DETERMINATIONS

Date	Water	Reducing sugar	Invert sugar	Total sugar	Naringin	Hydrolyzable polysaccharides	Soluble solids	Insoluble solids	Hydrogen-ion concentration of rind	Flavado-albedo ratio	Hydrogen-ion concentration of pulp juice	Milli-liter N/1 acid in 10 ml pulp juice
	Percent	Percent	Percent	Percent	Percent	Percent	Percent	Percent	pH	Percent	pH	
1933												
Nov. 6	74.1	14.23	7.56	21.79	9.21	14.86	52.6	47.4	5.74	0.79	3.28	2.69
Dec. 11	74.4	13.55	9.24	22.79	7.21	12.92	53.1	41.9	6.01	.86	3.20	2.47
1934												
Jan. 15	75.1	27.64	7.69	34.73	4.93	10.59	54.7	35.3	6.26	.83	3.26	2.50
Mar. 1	80.1	22.60	9.95	32.64	5.76	10.85	63.1	36.9	4.71	.83	3.22	2.61
Apr. 9	80.3	20.38	14.00	34.38	5.26	8.87	65.7	33.3	6.46	.96	3.47	2.11

<sup>1</sup> All values except those in the second and the last 4 columns are calculated on the basis of dry weight.

Soluble solids increased notably until the fourth collection, when they reached a maximum. Their graph (fig. 1) was similar to that of total sugar, which accounted for slightly more than half of the soluble solids.

Naringin (fig. 2) fluctuated considerably in various samples, but on the whole decreased during the season.

Hydrolyzable polysaccharides (fig. 2) diminished slowly throughout the season, or at least became relatively less.

Hydrogen-ion concentration in the rind changed very slightly, but there appeared to be an increase until the third collection (June 11), after which there was a small decrease, so that the pH was nearly the same at the time of the first and the last collections (fig. 2).

The average hydrogen-ion concentration of the pulp juice did not parallel that of the rind. The pH graph for pulp juice is presented in figure 2, and the titration values for the same juice samples are shown in table 1.

The flavedo-albedo ratio rose slowly until the July 23 collection and then decreased to a value less than that at the beginning of the season.

#### AT OASIS

A summary of the seasonal changes in the rind of the Oasis grapefruit is presented in table 1 and in figures 3 and 4. Each percentage value is the average of eight determinations.

Water increased slightly through the season, regardless of the fact that the weather was growing warmer and drier. The average relative humidity in the Coachella Valley for March and April 1934 was about 30 percent.

Soluble solids and total sugar (fig. 3) increased rather steadily through the season. The behavior of the total sugar was largely that of the reducing sugar until invert sugar increased greatly toward the end of the season.

Reducing sugar increased until January 15 and then decreased to April 9, whereas invert sugar increased slightly to December 11, then

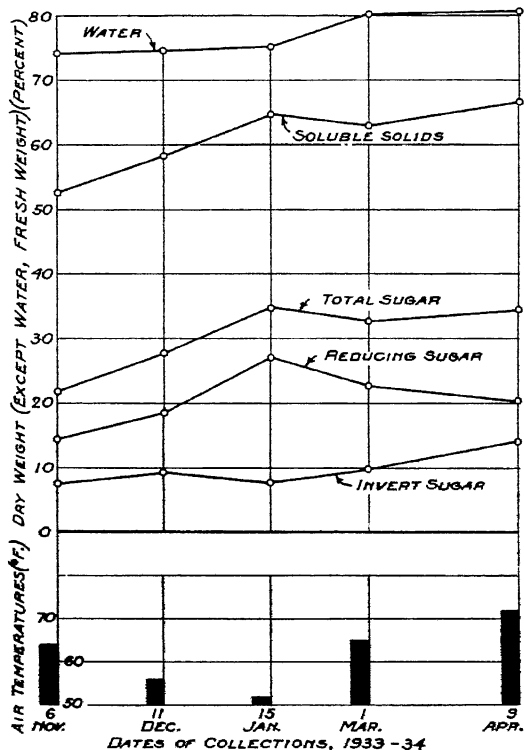


FIGURE 3.—Seasonal changes in mean air temperature (means for 5-day periods preceding collections) and in the composition of the rind of Marsh grapefruit from Oasis, Calif., with respect to water, soluble solids, and reducing, invert, and total sugars

decreased to January 15, and increased during the rest of the season. The seasonal graphs in figure 3 show that these sugars when compared with graphs for mean air temperature behaved very much as did the same sugars in the rind of the Fontana-Corona grapefruit (fig. 1) when their graphs were compared with block graphs for mean air temperature.

The hydrolyzable polysaccharides (fig. 4) showed a relative decrease through the season.

Naringin showed a general decrease, but there were fluctuations in naringin content resembling those noted in the rinds at Fontana and Corona. It seems very probable that naringin is an indirect by-

product of respiration. If so, there should be a tendency for naringin content to parallel the rate of respiration. Phloridzin, a closely related glucoside, has been found to behave so in apple shoots (11, 12). If the naringin situation is somewhat comparable, one may expect a general seasonal decrease of naringin while the fruit is attached to the tree and the rate of respiration becomes slower and translocation or destruction of naringin continues. But local conditions which speed up respiration might temporarily offset the general seasonal tendency of the glucoside to diminish. The naringin graphs (figs. 2 and 4) show fluctuations which may be tentatively explained in this

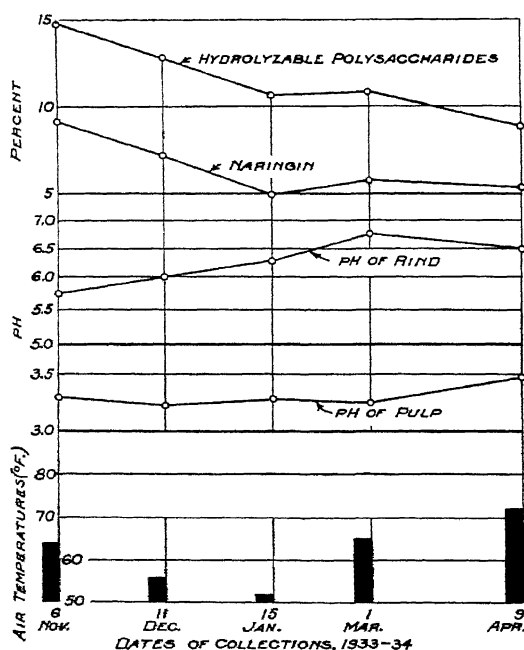


FIGURE 4.—Seasonal changes in mean air temperature (means for 5-day periods preceding collections) and in the composition of the rind of Marsh grapefruit from Oasis, Calif., with respect to naringin, hydrolyzable polysaccharides, and hydrogen-ion concentration. The pH of the pulp is also shown.

manner by noting the larger variations in the air temperature.

The decrease in the average hydrogen-ion concentration of the Oasis grapefruit rind was equivalent to about one pH unit during the season. The average hydrogen-ion concentration in the pulp juice also decreased, but the change was very slight.

The flavedo-albedo ratio changed as much as 21 percent, but the changes were rather irregular.

It is interesting to note that the seasonal changes in the rind of grapefruit just recorded agree in general with the findings of investigators who have studied seasonal changes in the pulp or the juice of grapefruit. In Florida, Collison (5) found that both reducing and

invert sugars increased in the juice while the grapefruit was attached to the tree, but that the changes were not so marked as in oranges. Invert sugar began to break down toward the end of the season. Acid also decreased gradually from the beginning of the season. Lopez-Dominguez (17) made the following observations on seasonal changes in the juice of grapefruit grown in Puerto Rico: Total solids remained fairly constant, acid decreased steadily; total sugar increased gradually to a solids-acid ratio of 7 and then fluctuated; and invert sugar increased throughout the season. These results are in general agreement with the findings of Hawkins (14) for Florida grapefruit.

## COMPARISON OF STEM AND BLOSSOM ENDS

## AT FONTANA AND CORONA

The data presented in table 2 have been arranged for comparing the stem-end and blossom-end portions of the rind of grapefruit from Fontana and Corona. The differences in composition recorded for these rind portions were surprisingly small in view of the very much larger corresponding differences noted in the studies on Valencia and Washington Navel orange rinds (13).

The stem-end rind had more water, reducing sugar, invert sugar, and soluble solids than the blossom end. The latter was higher in hydrolyzable polysaccharides and hydrogen-ion concentration, but, as mentioned above, none of the differences is large, and perhaps some are of questionable significance in spite of the large number of analyses involved.

TABLE 2.—*A comparison of the chemical composition of Marsh grapefruit stem-end and blossom-end rinds at Corona and Fontana and at Oasis, Calif., during the season of 1933-34*<sup>1</sup>

## CORONA AND FONTANA, 1933

Portion of rind	Water		Reducing sugar		Invert sugar		Total sugar		Naringin	
	Average	Range	Average	Range	Average	Range	Average	Range	Average	Range
Stem end.....	Pct. 81.5	Pct. 80.3-82.5	Pct. 25.23	Pct. 22.70-26.82	Pct. 12.18	Pct. 8.35-17.00	Pct. 37.41	Pct. 35.16-39.69	Pct. 7.31	Pct. 4.62-8.31
Blossom end....	Pct. 78.2	Pct. 72.9-79.5	Pct. 24.62	Pct. 23.41-26.27	Pct. 11.50	Pct. 7.85-15.59	Pct. 36.13	Pct. 34.12-39.00	Pct. 7.32	Pct. 5.51-8.38

Portion of rind	Hydrolyzable polysaccharides		Soluble solids		Insoluble solids		Hydrogen-ion concentration		Flavedo-albedo ratio	
	Average	Range	Average	Range	Average	Range	Average	Range	Average	Range
Stem end.....	Pct. 7.32	Pct. 6.03-9.68	Pct. 70.2	Pct. 65.0-73.3	Pct. 29.8	Pct. 26.7-35.0	pH <sup>1</sup> 6.45	pH 6.25-6.66	Pct. 0.74	Pct. 0.69-0.78
Blossom end....	Pct. 7.45	Pct. 6.11-9.12	Pct. 66.5	Pct. 63.2-74.1	Pct. 30.5	Pct. 25.9-34.8	pH 6.40	pH 6.29-6.59	Pct. .75	Pct. .65-.81

<sup>1</sup> All values except those for the water, hydrogen-ion concentration, and flavedo-albedo ratio are calculated on the basis of dry weight.

<sup>2</sup> The writers are fully aware that the averaging of pH values, here and elsewhere in this paper, is mathematically unsound, but they believe that through the ranges shown the averages of pH values as numerical entities are probably as sound biologically as the averages of the actual hydrogen-ion concentrations or the equivalent pH values, and are considerably more convenient.

TABLE 2.—A comparison of the chemical composition of Marsh grapefruit stem-end and blossom-end rinds at Corona and Fontana and at Oasis, Calif., during the season of 1933-34—Continued

OASIS, 1933-34

Portion of rind	Water		Reducing sugar		Invert sugar		Total sugar		Naringin	
	Average	Range	Average	Range	Average	Range	Average	Range	Average	Range
Stem end.....	Pct. 77.4	Pct. 74 7-81.2	Pct. 20.73	Pct. 14 95-27.42	Pct. 9.04	Pct. 7.09-12.60	Pct. 29.77	Pct. 22.29-34.51	Pct. 6.76	Pct. 4.68-9.84
Blossom end....	76.3	73 0-80.1	20.42	13.50-26.65	10.33	7.77-15.39	30.75	21.27-36.61	6.17	4.69-8.57

Portion of rind	Hydrolyzable polysaccharides		Soluble solids		Insoluble solids		Hydrogen-ion concentration		Flavedo-albedo ratio	
	Average	Range	Average	Range	Average	Range	Average	Range	Average	Range
Stem end.....	Pct. 11.92	Pct. 9 44-15.50	Pct. 60.9	Pct. 51 4-65.4	Pct. 39.1	Pct. 34.6-48.6	pH <sup>1</sup> 6.24	pH 5.78-6.79	Pct. 0.81	Pct. 0.75-0.88
Blossom end....	11.34	8 30-14.26	61.2	53.7-68.3	38.8	31.7-46.3	6.22	5.70-6.61	.89	.82-1.04

<sup>1</sup> The writers are fully aware that the averaging of pH values, here and elsewhere in this paper, is mathematically unsound, but they believe that through the ranges shown the averages of pH values as numerical entities are probably as sound biologically as the averages of the actual hydrogen-ion concentrations or the equivalent pH values, and are considerably more convenient.

Naringin and the flavedo-albedo ratio were approximately the same in both rind portions.

The total average difference for all substances in the two ends was 3.4 percent. The corresponding average differences in the Valencia and Washington Navel orange rinds were about 13 and 11 percent, respectively.

## AT OASIS

Table 2, which also presents the data for the stem and blossom ends of the rind of Marsh grapefruit from Oasis, shows that the difference between the two ends was even less than that in the rind of grapefruit from the Fontana-Corona district. The total average difference was only 1.3 percent.

## COMPARISON OF FLAVEDO AND ALBEDO

Table 3 shows the chemical composition of the flavedo and albedo of grapefruit from Fontana and Corona. The differences in composition of these tissues were very small, the total average difference being only 2.2 percent. The corresponding difference between the flavedo and albedo for Valencia and Washington Navel oranges (13) had been found to be about 10 percent and 16 percent, respectively.

Table 3 also offers a comparison of the flavedo and albedo of grapefruit from Oasis. The two tissues show significant differences in regard to some substances. The flavedo was considerably higher than the albedo in invert sugar, total sugar, and soluble solids. The albedo was higher in naringin, hydrolyzable polysaccharides, and hydrogen-ion concentration. The total average difference was 8.3 percent, or nearly equal to the corresponding difference in Valencia oranges (13).

TABLE 3.—*A comparison of the chemical composition of grapefruit flavedo and albedo at Corona and Fontana, and at Oasis, Calif., during the season of 1933-34*<sup>1</sup>

## CORONA AND FONTANA, 1933

Portion of rind	Water		Reducing sugar		Invert sugar		Total sugar		Naringin	
	Average	Range	Average	Range	Average	Range	Average	Range	Average	Range
Flavedo.....	Pct. 79.4	Pct. 77.5-80.8	Pct. 25.00	Pct. 22.70-27.10	Pct. 11.79	Pct. 7.93-15.75	Pct. 36.79	Pct. 35.03-39.04	Pct. 6.38	Pct. 4.17-7.87
Albedo.....	Pct. 80.3	Pct. 79.6-80.9	Pct. 24.85	Pct. 22.74-26.23	Pct. 11.89	Pct. 8.27-16.84	Pct. 36.74	Pct. 34.26-38.39	Pct. 8.26	Pct. 5.96-10.35
Portion of rind	Hydrolyzable polysaccharides		Soluble solids		Insoluble solids		Hydrogen-ion concentration		Flavedo-albedo ratio	
	Average	Range	Average	Range	Average	Range	Average	Range	Average	Range
Flavedo.....	Pct. 6.26	Pct. 5.42-7.48	Pct. 71.4	Pct. 68.3-74.8	Pct. 28.6	Pct. 25.2-31.7	pH 6.72	pH 6.52-6.94	Pct. 0.74	Pct. 0.71-0.77
Albedo.....	Pct. 8.51	Pct. 6.88-11.31	Pct. 68.3	Pct. 61.9-72.5	Pct. 31.7	Pct. 27.5-38.1	pH 6.14	pH 5.96-6.30		

## OASIS, 1933-34

Portion of rind	Water		Reducing sugar		Invert sugar		Total sugar		Naringin	
	Average	Range	Average	Range	Average	Range	Average	Range	Average	Range
Flavedo.....	Pct. 76.7	Pct. 72.7-81.3	Pct. 20.06	Pct. 11.58-27.73	Pct. 10.94	Pct. 5.37-15.26	Pct. 31.00	Pct. 16.95-36.15	Pct. 4.80	Pct. 3.01-8.24
Albedo.....	Pct. 77.0	Pct. 74.6-80.0	Pct. 21.09	Pct. 16.87-26.33	Pct. 8.43	Pct. 5.01-12.73	Pct. 29.52	Pct. 25.52-32.60	Pct. 8.16	Pct. 6.85-10.17
Portion of rind	Hydrolyzable polysaccharides		Soluble solids		Insoluble solids		Hydrogen-ion concentration		Flavedo-albedo ratio	
	Average	Range	Average	Range	Average	Range	Average	Range	Average	Range
Flavedo.....	Pct. 10.27	Pct. 7.46-14.09	Pct. 64.3	Pct. 53.8-70.1	Pct. 35.7	Pct. 29.9-46.2	pH 6.54	pH 5.89-7.07	Pct. 0.81	Pct. 0.75-1.04
Albedo.....	Pct. 12.90	Pct. 10.23-15.67	Pct. 57.7	Pct. 51.3-63.4	Pct. 42.3	Pct. 36.6-48.7	pH 5.93	pH 5.60-6.34		

<sup>1</sup> All values except those for the water, hydrogen-ion concentration, and flavedo-albedo ratio are calculated on the basis of dry weight.

It is interesting to note that the grapefruit from Oasis showed less difference between the stem-end and blossom-end rinds and more difference between flavedo and albedo than did the grapefruit from Fontana and Corona. The same relation was noted in a similar comparison between Valencia and Washington Navel orange rinds (13).

## COMPARISON OF FRUIT FROM DIFFERENT LOCALITIES

Table 4 presents a comparison of the composition of grapefruit rinds from the three localities from which fruits were obtained. The

Fontana and Corona values were averaged, as shown in the third line of table 4, in order to be in agreement with the other comparisons in this study.

TABLE 4.—Comparison of the chemical changes in the rind of Marsh grapefruit in different localities during the season of 1933<sup>1</sup>

Locality	Water	Reducing sugar	Invert sugar	Total sugar	Naringin	Hydrolyzable polysaccharides	Soluble solids	Insoluble solids	Hydrogen-ion concentration	Flavedo-albedo ratio
	Percent	Percent	Percent	Percent	Percent	Percent	Percent	Percent	pH	Pct.
Corona.....	80 00	24 02	12 62	36 64	7 64	7 40	70 00	30 00	6.30	0.77
Fontana.....	79.70	25.83	11.07	36.90	6.98	7.37	69.70	30.30	6.55	.71
Average.....	79.85	24.93	11.85	36.77	7.31	7.39	69.85	30.15	6.43	.74
Oasis.....	76.80	20.58	9.69	30.27	6.47	11.63	61.00	39.00	6.24	.81

<sup>1</sup> All values except those for water, hydrogen-ion concentration, and flavedo-albedo ratio are calculated on the basis of dry weight.

When the individual values of the three localities are examined separately, it becomes evident that the Fontana and Corona rinds agree more closely in composition than either agrees with the Oasis rinds. This fact tends to justify the present plan of handling the Fontana and Corona data together and contrasting them with the Oasis data. Of the two former, however, the Corona showed a slightly closer resemblance to the Oasis rinds.

The average of the Fontana and Corona rinds was higher than that of the Oasis rinds in water, reducing sugar, invert sugar, naringin, and soluble solids. This higher sugar content of the Fontana and Corona rinds was rather surprising. The Oasis rinds were higher in hydrolyzable polysaccharides and hydrogen-ion concentration, and had a larger flavedo-albedo ratio.

#### DISCUSSION

In the foregoing presentation of data on the composition of the Marsh grapefruit rind consideration has been given to seasonal changes, differences between stem and blossom ends, flavedo and albedo, and the possible influence of local or climatic environment. Measurable changes and differences were noted in most of these comparisons. In this and in another report on citrus rinds (13), the writers have placed some emphasis upon such differences because of the possible relation of some of them to subsequent pitting and spotting of citrus rind in storage or during transportation. For example, it has been pointed out that if the stem-end rind differed considerably in composition from that of the blossom end and was much more subject to spotting, then those differences in composition might be associated with the cause or causes of the trouble. Again, corresponding differences in the composition of the flavedo and albedo might be of importance in determining why pitting or spotting originated in one or the other of these tissues.

In the comparison of stem- and blossom-end rinds, it was noted not only that grapefruit showed less difference between these portions than did oranges, but also that while the difference was still noticeable in the Fontana-Corona rinds it was very small in the Oasis rind. But in the comparison of flavedo and albedo it was noted that the difference between these tissues was greater at Oasis than at Fontana



and Corona, and less in rinds of fruit from all these localities than in the orange rinds.

That there can be no hard and fast interpretation of such differences as may be recorded in total seasonal averages seems obvious. But there may be something of considerable value, theoretically, in observing variations in the differences between the various tissues in relation to changes in the season and in the immediate environment.

The matter of this seasonal difference between rind tissues has been discussed in the report on orange rinds (18). It is referred to again because the graphs representing these seasonal differences (figs. 5 and 6) show how extremely variable are the relations in regard to composition between any two tissues of the rinds under comparison.

The average value of the difference for the season as a whole is often small as compared with differences that may appear at various times during the season. Changes may occur in the rind at a given period

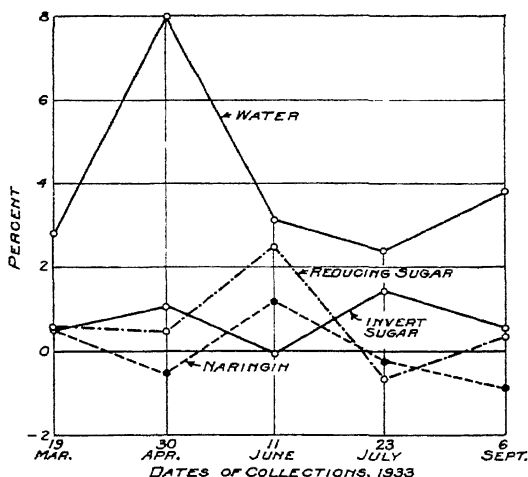


FIGURE 5.—Seasonal changes in the differences between the average composition of the stem end and blossom end of the rind of Marsh grapefruit from Corona and Fontana, Calif., with respect to water, reducing and invert sugar, and naringin. Positive values indicate the stem end higher than the blossom end and negative values the reverse.

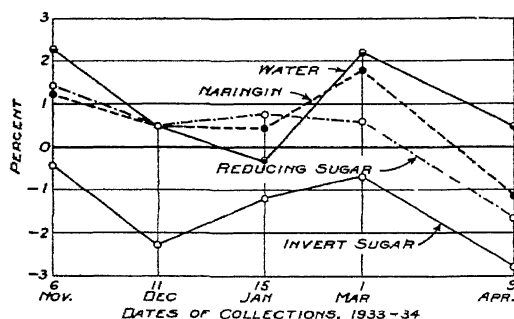


FIGURE 6.—Seasonal changes in the differences between the average composition of the stem end and blossom end of the rind of Marsh grapefruit from Oasis, Calif., with respect to water, reducing and invert sugar, and naringin. Positive values indicate the stem end higher than the blossom end and negative values the reverse.

that are a complete reversal of the trend as indicated by the seasonal averages. The Fontana-Corona average seasonal difference in the two ends was recorded as 3.4 percent, yet the graphs of figure 5 show generally less than this figure. The much larger percentage of water in the stem end helped greatly to increase the seasonal average difference. At the second collection the difference in water content between the rind of the ends of the fruit was about 5 percent greater than the normal seasonal difference. Naringin was higher in the stem end at the first and third collections, but lower at the second, fourth, and fifth collections. Reducing sugar, usually higher in the stem end, was lower at the fourth collection.

At Oasis, where the average seasonal difference between stem end and blossom end was 1.3 percent, an amount scarcely considered significant, figure 6 shows that there were many variations in respect to individual differences. The tissues show a different relation to each other in respect to composition at every collection date. Naringin, which was higher in the stem end through four collections, was lower at the fifth collection. Water and sugars varied constantly in their interrelationships.

The foregoing data indicate that, when it is said that one portion of the rind of a citrus fruit contains more of a certain substance than another, it should be made plain whether one is speaking generally or of specific instances. Apparently almost any relationship can be found between rind tissues on occasion, and these occasional changes may have much to do with the subsequent behavior of citrus rinds. It seems clear that the interrelationships of substances in the various portions of the rind can be modified relatively rapidly and significantly by weather conditions.

### STORAGE STUDIES

So far no reference has been made to findings that might relate time of picking, composition of rind, location, etc., to pitting and spotting of grapefruit in storage. The rinds used in the studies just reported were analyzed directly from the field, and therefore had no opportunity to develop spotting. However, for each lot of fruit used in the field studies a corresponding lot was collected and placed in storage. At the end of the storage periods these were inspected and later analyzed. The principal aims of these studies were (1) to determine some of the general chemical changes occurring in grapefruit rinds during storage, (2) to note the relation of such changes to the previously determined condition of the rinds at the time of picking, (3) to correlate the findings with the susceptibility of the rinds to pitting and spotting, and (4) to study the effects of various substances and treatments on pitting and spotting.

### MATERIALS AND METHODS

The grapefruit used in the main storage experiments were from the same selected Marsh grapefruit trees that furnished the material for the studies of rinds in the field. At the time that most of the field collections of grapefruit for immediate study were made, additional lots were taken for storage under different conditions. The relation of the former to the latter and to the season is shown in figure 7.

The storage temperatures employed were 32° to 33°, 42°, and 51° to 52° F.; they fluctuated through a relatively narrow range, the greatest variation being in the 32° to 33° room. Throughout the paper the temperatures are referred to simply as 32°, 42°, and 52°, unless there is need to mention the exact temperature. In the three storage rooms an average relative humidity of 84, 87, and 89 percent, respectively, was maintained. The length of the storage periods was generally about 6 weeks. At the end of that time inspections were made for pitting and spotting, and the rinds were prepared for analysis.

The methods of preparing and analyzing the rinds were identical with those described in previous reports (13, 14, 24). As in the field

studies, the rind was separated into four parts, as follows: Stem-end flavedo; stem-end albedo; blossom-end flavedo; and blossom-end albedo. The analyses of the rinds included the determination of water, soluble and insoluble solids, reducing sugar, invert sugar, naringin, hydrolyzable polysaccharides, and hydrogen-ion concentration. The juice from the pulp was examined only for hydrogen-ion concentration and titratable acidity.

#### MAIN STORAGE EXPERIMENTS

Two collections for storage comprising three lots each were made during the season at Corona and Fontana, and five were made at

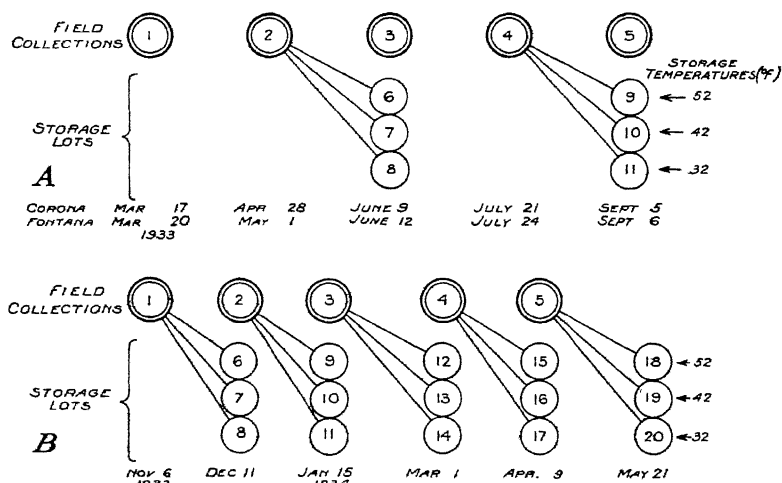


FIGURE 7.—Diagram showing relationship of storage lots to the various field collections: A, Corona and Fontana; B, Oasis.

Oasis. In fact, at the latter location there was a collection for storage corresponding to every field collection for immediate use.

The results of the investigations of the rinds of these grapefruit after storage will be presented separately for the two principal districts involved. The Corona and Fontana grapefruit rinds behaved very much alike qualitatively in almost every detail noted. On the other hand, the behavior of the rinds of the Oasis fruit differed in several respects from those of Corona and Fontana.

#### DESCRIPTION OF THE STORAGE LOTS AND INSPECTIONS

The first Corona and Fontana storage lots correspond to the second immediate field collections picked April 28 and May 1 in the two respective locations and placed directly in storage. The fruit was firm and well-colored when picked, but could not be considered further advanced than early maturity. At the time of removal from storage the fruit was still firm in all three storage rooms. The effects of temperature and other storage conditions on pitting and spotting of the rinds are presented in table 5.

TABLE 5.—Effect of storage temperature and date of picking on pitting and spotting of Marsh grapefruit grown at Corona and Fontana and at Oasis, Calif., 1933-34

## CORONA AND FONTANA, 1933

Date of collection	Location	Lot no.	Storage period	Storage temperature	Relative humidity		Pitting and spotting		
					Weighted average	Range	Slight <sup>1</sup>	Severe <sup>1</sup>	Total
			Days	° F	Percent	Percent	Percent	Percent	Percent
1933 Apr. 28	Corona.....	6	42	51	88	84-92	5	0	5
		7	42	42	85	79-89	13	50	63
		8	42	32	84	83-90	30	0	30
July 21		9	46	52	87	82-94	16	0	16
		10	46	42	85	78-89	30	33	63
	Fontana.....	11	46	33	79	75-85	10	7	17
May 1		b	42	51	88	84-92	5	0	5
		7	42	42	85	79-89	24	76	100
		8	42	32	84	83-90	3	27	30
July 24		9	44	52	87	82-94	10	0	10
		10	44	42	85	78-89	31	60	91
		11	44	33	79	75-85	51	21	72

## OASIS, 1933-34

Nov. 6	Coachella Valley.....	6	33	52	89	87-94	0	0	0
		7	33	42	87	83-91	0	100	100
		8	33	33	86	79-91	0	0	0
Dec 11		9	37	52	94	92-95	0	0	0
		10	37	42	86	76-90	25	25	50
1934 Jan. 15		11	37	32	81	76-89	0	0	0
		12	44	52	89	80-90	0	0	0
		13	44	42	88	79-91	22	8	30
		14	44	33	88	82-93	6	12	18
Mar. 1		15	39	51	89	84-93	0	0	0
		16	39	42	89	85-92	34	8	42
		17	39	33	88	83-92	35	18	53
Apr. 9		18	42	52	90	86-94	0	0	0
		19	42	42	93	91-95	64	28	92
		20	42	33	89	85-91	54	44	98

<sup>1</sup> Slight pitting and spotting includes only such cases as were not considered of commercial importance. Severe pitting and spotting includes all cases which might affect the commercial value of the fruit. A rather arbitrary standard involving both size and number of spots had to be adopted for such judgments.

The second Corona and Fontana storage collections (lots 9 to 11) correspond to the fourth field collections for immediate study. They were picked July 21 and July 24 at the two respective locations and placed in storage at once. The fruit when picked was somewhat less firm than the previous collection because of its considerably advanced maturity and the prevailing warmer weather. It was also less firm at the end of the storage period, but was not seriously shrunken. The condition as regards pitting and spotting is given in table 5.

The first Oasis storage collection was picked November 6 and placed in storage the same day. It corresponds to the first field collection for immediate study. The fruit was rather dark green, and the date of picking preceded the first commercial picking by about 3 weeks. The fruit was firm when removed from storage, and that which had been at 52° F. was beautifully colored and entirely free from storage blemishes. That from 42° was severely pitted and spotted, and in the occasional areas free from these blemishes there was considerable green color remaining. That from 32° was almost as green as when placed in storage, but entirely free from storage pits or spots. Plate 1, A-C, shows the appearance of these lots after storage. The results are given numerically in table 5.

The second Oasis storage collection was picked December 11 and placed in storage the same day. The color of the fruit was light green, and commercial picking had been in progress about 3 weeks. On removal from storage the fruit that had been held at 52° F. was a clear yellow and entirely free from storage blemishes; that held at 32° still retained some green color but was also free from blemishes; but that held at 42° was badly pitted and spotted, although less so than the corresponding first storage collection (table 5).

The third Oasis storage collection was picked January 15 and placed in storage the same day. The fruit was fully colored and firm, and represented prime harvest maturity. At the end of the storage period it was the least pitted and spotted of any used in the Oasis storage experiments (table 5).

The fourth Oasis storage collection was picked March 1 and stored the same day. The fruit was not so firm as that of previous collections, but still was in very good condition. In storage the spotting had become rather serious at 32° F. and the injury had increased somewhat at 42° (table 5).

The fifth Oasis storage collection was picked April 9 and stored the same day. The fruit was noticeably past prime harvest condition. The effects of higher air temperatures and advanced maturity were indicated by lack of firmness, large sizes, and "hollow cores." In storage this fruit pitted and spotted almost as badly as the November collection (table 5), although the number of "severe" cases was not so large. The response of the Oasis grapefruit rinds to the storage conditions furnished is shown in figure 8.

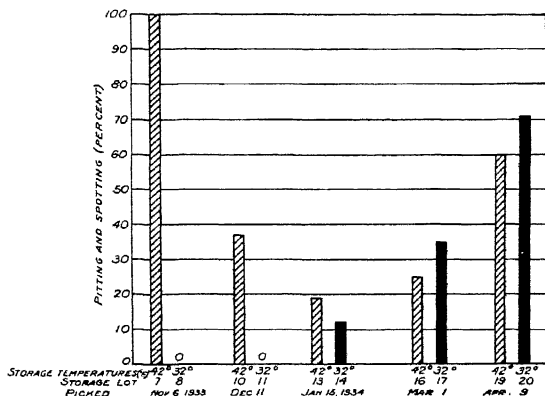


FIGURE 8.—Seasonal changes in susceptibility to pitting and spotting of Marsh grapefruit from Oasis after storage at 32° and 42° F. No pitting or spotting at 52°

#### RELATION OF PITTING AND SPOTTING TO TIME OF PICKING AND TEMPERATURE OF STORAGE

The variation in the effects of the different storage temperatures with the season of picking was very interesting. In the fruit from Oasis at 52° F. there was no pitting or spotting of grapefruit picked at any stage of maturity; but at 42° every fruit of the November collection was severely affected with storage spots. The December collection had about one-half as much spotting as the first, but not all the fruits involved were seriously affected. The January collection showed the least susceptibility to spotting but in later collections spotting increased greatly.

The same variation in susceptibility to storage spotting with variations in temperature and stage of maturity was indicated by the behavior of the two storage collections from Corona and Fontana. The

first collection from both localities showed a very high susceptibility to spotting at 42° F., but low at 32°, but in the second collection spotting increased greatly at 32° in the Fontana fruit. There were only traces of pitting and spotting at 52° in any of the Corona and Fontana fruit. These two storage collections are believed to correspond as regards maturity to the second and fourth Oasis collections.

It should be emphasized that the types of spotting or pitting which occurred at 42° and 32° F. were noticeably different. The spotting or pitting at 32° usually involved very small islands of tissue. These were similar to the characteristic "pock" or the small, sharply sunken, but almost colorless blemishes commonly occurring on California grapefruit exported to England. Another type of spotting at 32° was small, shallow, and reddish in color. In one lot at 32° there developed a sort of soft scald. The spotting or pitting occurring at 42°, however, usually involved large irregular areas which were more or less discolored and often showed concentric rings marking their development. In addition, coalescence of spots frequently occurred.

When the grapefruit was brought from storage at 32° to 33° F. and held either at room temperature or at 52° for a few days, it usually developed severe spotting. These spots resembled the characteristic 42° rather than the 32° spots.

Taking the results of the storage tests as a whole, there is little doubt that a temperature of 40° to 42° F. produced more spotting of California Marsh grapefruit than the other temperatures used. This conclusion seems to be corroborated by the unpublished observations of a number of other workers. Although temperatures below 42° may not produce so much spotting in storage, they tend to render grapefruit more susceptible to spotting after removal to higher temperatures.

For more than a decade workers (1, 8, 9, 14, 15, 16, 19) in various parts of the country have attempted to determine the best storage conditions for grapefruit. Such investigations have not led to a general agreement, and at present it seems very improbable that there will ever be a satisfactory recommendation that grapefruit should be held at a certain definite temperature and relative humidity. The situation is too complex to have a simple solution. Some of the important factors to be taken into consideration are variety, the section of the country where the fruit is grown, the districts within those sections, the cultural treatment of the grove, stage of maturity of the fruit, weather conditions at time of picking, and the various packing-house treatments. The importance of most of these factors is now recognized and some effort is being made to integrate them sufficiently to allow special and practical recommendations to be made, at least for the different citrus-growing districts. The readiness with which grapefruit from the different districts become infected by mold and other organisms may sometimes require the recommendation of a storage temperature known to be far from ideal for the best control of storage spotting. The same procedure may be necessary in order to avoid the development of too deep a color in storage at the higher temperatures.

Considering only the physiology of California Marsh grapefruit rinds as regards spotting and pitting in storage, there remains little doubt that a temperature of 50° to 55° F., or somewhat higher, and a relative humidity of approximately 90 percent would be much better than anything below these values. These results are in agreement with those of Brooks and McCulloch (1) for Florida grapefruit.

## CHEMICAL ANALYSIS

## CORONA AND FONTANA

In table 6 are summarized the chemical changes recorded for the Corona and Fontana grapefruit rinds in storage at different temperatures. For each storage temperature the data presented are the combination of both storage collections from the two localities and for all portions of the rind. Each figure in the table is the average of 32 determinations.

TABLE 6.—*Effect of storage temperature on the composition of the rind of Marsh grapefruit grown at Corona and Fontana, Calif.*

[Percentages except water are on dry-weight basis, each figure being the average of 32 determinations]

Item	No storage	52° F.	42° F.	32°–33° F.
Water.....Percent.....	80.1	79.1	79.6	79.5
Reducing sugar.....do.....	24.58	29.52	30.33	23.85
Invert sugar.....do.....	12.91	2.69	3.60	11.40
Total sugar.....do.....	37.49	32.21	33.93	35.25
Naringin.....do.....	8.03	7.40	6.91	6.88
Hydrolyzable polysaccharides.....do.....	7.28	7.56	7.41	7.44
Soluble solids.....do.....	70.5	68.1	68.4	69.6
Insoluble solids.....do.....	29.5	31.9	31.6	30.4
Flavado-albedo ratio.....do.....	.76	.76	.71	.72
Rind.....pH.....	6.40	6.10	5.97	6.29
Pulp juice.....pH.....	3.09	3.08	3.06	3.09
Acidity <sup>1</sup> .....ml.....	3.33	3.30	3.30	3.17

<sup>1</sup> As milliliters of N/1 acid per 10 ml of juice.

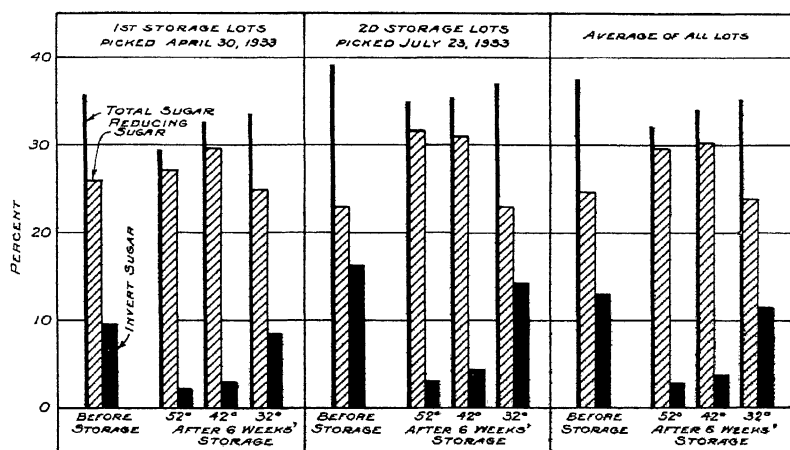


FIGURE 9.—Effect of storage temperature on the sugar content of the rind of Marsh grapefruit picked at different times; average of fruit from Corona and Fontana.

The water content of the rinds changed only slightly during storage, but tended to decrease. Total sugar invariably decreased, this decrease being least at 32° F. and greatest at 52°. This and other points referred to below are shown graphically in figures 9 and 10. Reducing sugar always increased at 52° and 42°, the increase being

about the same at both temperatures (fig. 9). At 32° there was little change in the amount of reducing sugar, although a slight decrease was noted.

Invert sugar decreased strikingly at all temperatures except at 32° F., at which the decrease was slight. Although the difference in temperature between the storage rooms was 10° the amount of inversion of sucrose<sup>3</sup> was nearly the same at 52° and 42° but very much less at 32°. Apparently there is some critical point lying between 32° and 42° where the action of invertase in the rind tissue is

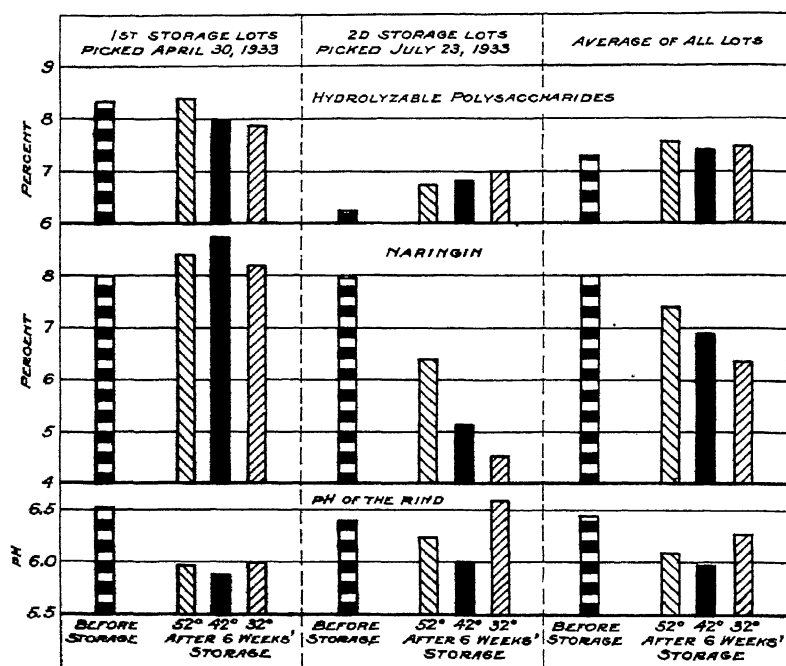


FIGURE 10.—Effect of storage temperature on hydrolyzable polysaccharides, naringin, and hydrogen-ion concentration of the rind of Marsh grapefruit picked at different times; average of fruit from Corona and Fontana.

strongly inhibited. Invertase, acting upon sucrose solutions in vitro at the three storage temperatures, did not show a corresponding break between 32° and 42°. At the two higher temperatures inversion apparently proceeded faster than the products were used in respiration—a supposition that accounts for the increase of reducing sugars noted at these temperatures. The same behavior of the different sugars in storage as outlined above was noted in every portion of the rind for both localities. The actual amount of inversion at the higher storage temperatures depended on the original sucrose content. The higher the latter the more the inversion, but the percentage of sucrose that remained was practically always the same regardless of the original content.

<sup>3</sup> The term "sucrose" has generally been avoided; however, the writers are certain that "invert sugar" of this report is derived almost wholly from sucrose.



Naringin in the Corona and Fontana rinds usually decreased at all temperatures (fig. 10), the decrease being greatest at 32° F. and least at 52°. Naringin, however, does not always decrease in storage. In a few instances it increased in the Corona and Fontana rinds, and as will later be shown, the synthesis of naringin in the Oasis rinds in storage was the usual occurrence. Zoller (24) reported a rapid hydrolysis of naringin in grapefruit during storage, but the conditions of storage were not given. It has since been stated repeatedly by Hawkins (14, 15) and others (7, 18) that naringin decreases in storage, but without indication that actual determinations of naringin had been made. The studies of the writers on the behavior of naringin and hesperidin, and those of the senior writer on phloridzin, have shown that in living tissue separated from the tree, the phenolic glucosides will be hydrolyzed or synthesized, depending upon certain conditions which are poorly understood. The difficulty of understanding seems to lie in the fact that a seemingly very slight change in the environment may determine the direction of the reversible reaction. The condition of the tissue when collected is apparently an important determinant of the subsequent behavior of the glucoside. In any case no one can say to date that naringin has decreased in storage without determining it in each instance.

Determinations of the hydrogen-ion concentration showed that active acidity increased, except in a few instances, at 32° F. At 42° the increase was greatest in such a large proportion of cases that the tendency may be considered as unmistakable. It was more or less expected that the increase in acidity would be least at 32° and greatest at 52°, but that it should be greatest at 42° was surprising.

During these studies a number of observations were made which indicate that the reactions going on in grapefruit rinds at 42° F. were very different from those at the other two temperatures. Of course, it is possible, and is sometimes indicated, that many of the same basic reactions may go on at 32° as at 42°, but if so they are probably unable to manifest themselves at the lower temperature. These facts appear to have special significance in view of the further fact that storage pitting or spotting was much more severe at 42°. However, it is not intended to imply that any of the chemical or physical reactions observed as happening at 42° are directly responsible for the storage pitting or spotting, although they may be associated in the same complex of reactions that cause the storage maladies.

The increased acidity at 42° F. is particularly interesting because high acidity was positively correlated with naringin content in all field and storage lots from every locality.<sup>4</sup> The higher the acidity the higher was the coefficient of correlation. This may indicate only that both naringin and active acidity are at times fairly good indexes of the relative rate of metabolism.

Hydrolyzable polysaccharides changed very slightly in any of the lots (fig. 10), but apparently they decreased during the storage period. Soluble solids gave about the same results.

A comparison of the chemical composition of the flavedo and albedo and of the stem and blossom ends of grapefruit rinds in the field was given earlier in this paper. The data from the present storage work

<sup>4</sup> The following coefficients of correlation of naringin and active acidity may be of interest: Field and storage samples of Corona and Fontana gave  $r=0.51 \pm 0.05$ ; storage only, Corona and Fontana,  $r=0.70 \pm 0.05$ ; and field and storage lots at Oasis,  $r=0.88 \pm 0.03$ .

showed the relations between the two tissues and the two end rinds to be so nearly like those described for the field samples used for immediate study that a separate presentation of a corresponding set of comparisons seems unnecessary.

The rinds of fruit from Corona and Fontana had much the same chemical composition and behaved similarly in storage. In general, the Corona rinds had slightly more invert sugar and less reducing sugar than the Fontana rinds. Active acid, naringin, and other substances examined were practically the same in the rinds from each locality. In storage the responses of these substances to the different temperatures were essentially the same. The rinds in the first storage experiment from both localities showed a slight tendency to synthesize naringin in storage, that is to say, naringin increased somewhat at 52° and 42° F. in the Corona rinds, but such differences are of doubtful importance, except as showing that small fluctuations

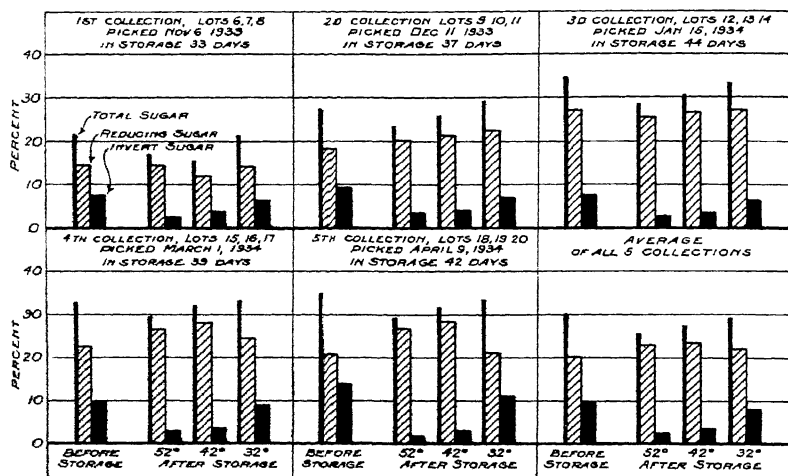


FIGURE 11.—Effect of storage temperature on the sugar content of the rind of Marsh grapefruit from Oasis.

were taking place in the equilibrium between naringin synthesis and hydrolysis. The possible consequences of a failure of the tissue to tie up naringenin, the derivative of naringin, is discussed later.

The relative susceptibility of the rinds of the two localities to pitting and spotting may be noted by referring again to table 5. Although the Fontana rinds show up a little worse than the Corona, the differences are such that macrochemical analyses cannot be expected to disclose what is really significant.

#### OASIS

The summarized data showing the changes that took place in the rinds of the Marsh grapefruit in storage, from Oasis, are presented in table 7. For each storage temperature the values given in the tables are the averages of all collections and all portions of the rind. Each figure is the average of 40 determinations.

Moisture conditions in the storage rooms were such that there was very little change in the water content of the rinds. At 52° F. there

was no appreciable change, and at 32° and 42° there was an average decrease of less than 1 percent during the storage period.

Total sugar decreased in storage at all temperatures (fig. 11).

TABLE 7.—*Effect of storage temperatures on the composition of Marsh grapefruit grown at Oasis, Calif.*

[Percentages except water are on dry-weight basis, each figure being the average of 40 determinations]

Item	No stor- age	52° F	42° F.	32° F.
Water.....Percent..	76.8	77.0	76.1	76.0
Reducing sugar.....do..	20.67	22.89	23.45	21.86
Invert sugar.....do..	9.69	2.69	3.65	8.06
Total.....do..	30.36	25.58	27.10	29.92
Naringin.....do..	6.47	7.73	7.95	8.17
Hydrolyzable polysaccharides.....do..	11.62	11.63	11.33	11.13
Soluble solids.....do..	61.0	58.9	59.8	61.7
Insoluble solids.....do..	39.0	41.1	40.2	38.2
Rind.....pH..	6.23	5.90	5.91	6.04
Pulp juice.....pH..	3.28	3.35	3.28	3.33
Acidity <sup>1</sup> .....ml..	2.48	2.56	2.50	2.43

<sup>1</sup> As ml of N/1 acid per 10 ml of pulp juice

Reducing sugar increased slightly at all storage temperatures (fig. 11 and table 7). The increase at 32° F. was largely at the expense of the polysaccharides, for the amount of inversion of sucrose at that temperature was probably not enough to satisfy the requirements for respiration. The increase of reducing sugar at the two other temperatures was due mostly to the rapid inversion of sucrose, for there was only a slight hydrolysis of polysaccharides at 42°. Although there was extensive inversion at 52°, the respiration at the higher temperatures prevented the increase in reducing sugar from exceeding the corresponding increase at 32°.

Invert sugar decreased at all storage temperatures (fig. 11). Inversion in the Oasis rinds was the same as in the Corona and Fontana rinds—that is, slight at 32° F. and rapid at 42° and 52°; also the rates were very similar at the last two temperatures.

Naringin increased at all temperatures (fig. 12). This synthesis of naringin was most pronounced at 32° F. and least at 52°.

It has been mentioned that naringin usually decreased in storage in the Corona and Fontana grapefruit rinds, and that the decrease was greatest at 32° F. The reversal of this entire situation at Oasis will be mentioned again, but it may be added here that the average change in naringin content of the different Oasis storage collections at all temperatures was in general in increasing order and that susceptibility to storage spotting was in the same but decreasing order with the exception of the fifth collection, which spotted nearly as badly as the first. The overmature fruit of the fifth collection behaved exceptionally in other ways, but the significance of the conditions which favor naringin synthesis in the rinds cannot be stated. The actual naringin content at the time of picking paralleled the susceptibility graph except for the fifth collection, when susceptibility increased and naringin decreased.

Soluble solids increased slightly at 32° F., but decreased at the other temperatures (table 7).

The active acidity of the rinds always increased in storage, most at 52° F. and least at 32° (fig. 12). The active acidity of the pulp juice in storage showed little change, but so far as could be noted it was downward.

The flavado-albedo ratio increased at the two highest temperatures, but decreased at 32° F. These changes in value of the ratio seemed to be due mainly to the albedo, which often showed a greater versatility in response to different conditions than the flavado.

#### OASIS COMPARED WITH CORONA-FONTANA

The data presented above deal entirely with the averages of all storage collections. Limitations of space prevent the presentation of the mass of data that would be required to show the size of deviations from the average condition that occurred in the rinds of individual

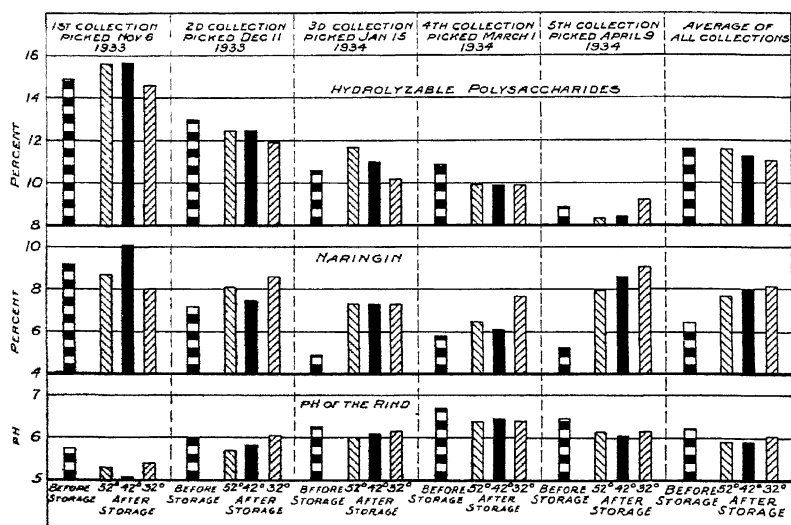


FIGURE 12.—Effect of storage temperature on hydrolyzable polysaccharides, naringin, and hydrogen-ion concentration of the rind of Marsh grapefruit from Oasis picked at different times.

collections or even in separate portions of the rinds in each collection. Figures 9 to 12, however, give a general idea of the variations between individual collections with respect to the substances represented. The first storage collection from each of the three localities showed a tendency to respond somewhat differently from the later collections. This is thought to have been due to the immaturity of the fruit at the time these collections were made, especially the first one at Oasis; otherwise a description of the behavior of the rinds of one collection would serve for the average of all. This statement is not so true for the separate portions of the rinds of individual collections in which there were a number of exceptions, but even for these it is true in the main. For example, naringin, which usually cannot be expected to behave in a very regular manner, not only showed a general tendency to be synthesized in the Oasis rinds in storage, but also responded in the same way in most of the portions of the rinds of all collections.

There were five exceptions in the stem-end rinds, three in the flavedo, and one in the albedo for all collections at all temperatures.

Total sugar in the Oasis rinds at the beginning of storage was about 7 percent less on a dry-weight basis (actually, not relatively) than in the Corona-Fontana rinds. This lower sugar content at Oasis was surprising, but the fact was tentatively attributed to the effect of conditions accompanying the winter harvest season as compared with the summer harvest season at Corona-Fontana. As compared with the fruit from the latter location, there was an average of more than 10 percent less water in the Oasis rinds at the beginning of storage; active acidity was also greater, but the naringin content was 1.50 percent lower. (See tables 6 and 7.) These and other differences in the original composition of the rinds may have been responsible for the differences subsequently noted in their behavior in storage.

Possibly the outstanding difference between the fruit from the two localities was the decrease of naringin in storage in the Corona-Fontana rinds and its corresponding increase in the Oasis rinds. The lower original sugar and naringin content, together with a higher active acidity in the Oasis rinds, might have been important factors in the observed synthesis of naringin in storage. The positive correlation between naringin content and active acidity was high, especially after storage, and this correlation was still higher in the above-average ranges of acidity in all collections and under all conditions of storage.

The hydrolyzable polysaccharides increased slightly in the Corona-Fontana rinds, but decreased at 42° F., and especially at 32°, in the Oasis rinds.

There were several other differences noted in the chemical responses of the grapefruit rinds from the two localities. The variations mentioned at least show that such tissues are capable of making opposite responses to a given experimental storage treatment and that the direction of the response is apparently determined by the internal condition of the tissue at the beginning of storage. These and other chemical responses recorded emphasize the possible variability of rind tissue and help toward a realization of the complexity of the problems encountered in the storage of citrus fruit.

#### MISCELLANEOUS STORAGE EXPERIMENTS

During the course of the main storage experiments an opportunity was found from time to time to carry out certain minor experiments suggested by apparent trends of the incoming data. However, because of the time elapsing between collections and the opportunity for examination of data from them, the larger number of these trends were observed too late to follow the leads with the same season's fruit. The minor experiments undertaken are reported below.

#### TRANSFER OF GRAPEFRUIT FROM ONE STORAGE TEMPERATURE TO ANOTHER

At the end of regular storage periods there were often extra lots of grapefruit which from time to time were transferred to a different temperature and held for further observation. The behavior of the rinds under such circumstances, as regards spotting, was sometimes rather striking. The previous storage conditions seemed to affect greatly the response to a second temperature. Of course this is a

situation quite generally known, but the details of such responses under given sets of conditions are of considerable interest. The only special undertaking by the writers to investigate this phase of the storage problem was one small series of storage experiments.

The grapefruit was collected at North Claremont, Calif., February 1, 1934, from a tree that belonged to a group previously selected to furnish fruit for other phases of the work. Nine lots of fruit were picked and placed in storage the same day at temperatures of 32°, 42°, and 52° F. Twenty-two days later all lots were inspected, then two lots from each storage room were transferred, one to each of the other two rooms. After 25 days more a final inspection was made. The results of both inspections are presented in table 8.

TABLE 8.—*Effects of transfer from one temperature to another on spotting of Marsh grapefruit in storage, using fruit grown at Claremont, Calif.*

Lot no.	Storage temperature	Spotting after 22 days			Lot no.	Temperature		Spotting after 25 days more		
		Severe	Slight	Total		From—	To—	Severe	Slight	Total
	°F.	Percent	Percent	Percent		°F.	°F.	Percent	Percent	Percent
1-----	52	0	0	0	1-----	52	52	0	0	0
2-----	52	0	0	0	2-----	42	52	15	19	34
3-----	52	0	0	0	3-----	32	52	0	15	15
4-----	42	0	28	28	4-----	42	42	18	19	37
5-----	42	0	31	31	5-----	52	42	0	5	5
6-----	42	0	15	15	6-----	32	42	10	29	39
7-----	32	0	0	0	7-----	32	32	14	14	28
8-----	32	0	0	0	8-----	52	32	0	5	5
9-----	32	0	10	10	9-----	42	32	29	24	53

It will be seen that there was very little spotting during the first storage period and that nearly all of it occurred at 42° F. No spotting was produced on the fruit held through both storage periods at 52°, and very little on the fruit held at 52° for the first storage period and then transferred to 42° and 32° for the second period. The fruit held at either 42° or 32° for the first period continued to spot after it was transferred to 52°. Lot 6, which was changed from 42° to 32°, fared somewhat the worst.

#### GAS STORAGE OF GRAPEFRUIT

In reporting the acidity changes observed in grapefruit rinds during storage, attention was called to the fact that in an overwhelming proportion of the determinations the active acidity was greatest in rinds stored at 42° F., as compared with those at 52° and 32°. Pitting and spotting of the rinds was also most prevalent at 42°. Without assuming any causal relationship between these two factors, it was thought worth while to undertake a few experiments to determine the possible effects of some volatile acids and bases and other substances on the pitting and spotting of grapefruit in storage, on the chance that the production of these blemishes might be accelerated or retarded, depending on the reaction of the atmosphere surrounding the fruit. The results of these experiments are reported below.

The first experiment was started June 26, 1933. Grapefruit was picked from one tree of a selected group at Fontana. Six lots of fruit were enclosed in large glass desiccators and held in storage at

52°, 42°, and 32° F., there being two lots at each temperature. One lot was exposed to the fumes from an acetic acid solution (100 ml of glacial acetic in 2.5 l of water); the other was kept over water as a check.

On July 6 the fruit at 42° F. in the acidified chamber showed well-defined beginnings of pitting. Two days later pitting and spotting had increased at 42°, but there was none in any of the other lots. The final inspection was made July 28, with the following results: At 52° there was no pitting or spotting either in the acidified or the check lot; at 42° four of the check fruits were slightly pitted, but the acidified fruit was all badly pitted around the stem end; at 32° the checks showed no pitting or spotting, but the acidified fruit showed a few small pits and traces of brown stain or scald.

The acidified atmosphere seemed clearly to have aggravated the pitting of the rinds, especially at 42° F. The pitting or spotting which followed acidification of the surrounding atmosphere appeared similar to the usual storage pitting and spotting.

The hydrogen-ion concentration of the rinds of the six lots was determined after the gross inspection. Of the three check lots, that from 42° F. showed the highest active acidity. The acidified lot at 42° gave approximately the same value, but the acidified lots at 52° and 32° were higher in acidity than their corresponding check lots, but not so high as either of the 42° lots.

A second gas-storage experiment was started July 28, 1933. The fruit was from the tree used in the first experiment, and in all other respects this experiment was a duplicate of the first.

By August 3 all acidified fruit held at 42° F. had begun to pit. No other lots were yet affected. On August 5 the acidified lot at 32° showed slight pitting, and the pitting had increased at 42°. Final inspection was made September 19. At 52° the check lot was firm and sound, but all the fruit of the acidified lot was badly pitted and spotted; at 42° nearly all the check lot was slightly pitted, but the acidified lot was entirely and very severely pitted and spotted; at 32° one check fruit was slightly pitted, but the acidified fruit was all severely pitted, although not so severely as at 42°. All the check fruit from 32° pitted and spotted severely within 24 hours after removal from storage. The general results of this second experiment were practically the same as those of the first.

A third gas-storage experiment was started August 10, 1933. The chambers used were earthenware jars of 3-gallon capacity. Panes of glass sealed tightly to the tops of the jars by means of modeling clay served as covers. The fruit was from the same source and the storage temperatures were the same as in the preceding gas-storage experiments. There were 10 lots which received the following treatments: 3 check lots over water only; 3 lots over acetic acid solutions (same strength as before); 3 lots over dilute ammonia solution (5 ml of 28-percent  $\text{NH}_3$  in 2.5 l of water); and 1 lot over a 6-percent ethyl alcohol solution. This last lot was stored at 42° F., and the others were distributed among the three storage temperatures. By August 21 the ammonified lots showed discolorations as a result of the too high concentration of ammonia, and the fruit was removed from these chambers and replaced with fresh fruit. The ammonia solution was then diluted to one-tenth its previous concentration. By

August 23 the acidified fruit at 42° and 52° had begun to pit. No other lots up to this time were affected.

The final inspection was made September 19, with the following results. At 52° F.: Check lot, one fruit slightly pitted; ammonified lot, all sound; acidified lot, all severely pitted and spotted. At 42°: Check lot, one fruit severely spotted; ammonified lot, four fruits slightly pitted; acidified lot, all fruits severely pitted and spotted; alcohol lot, four fruits slightly pitted. At 32°: Check lot, all sound; ammonified lot, all sound; acidified lot, all slightly to severely pitted or spotted.

The hydrogen-ion concentration of the rinds was determined after inspection. All lots were most acid at 42° F. The order of increasing acidity at every temperature was (1) ammonified, (2) checks, and (3) acidified. At 42° the lot over alcohol was the least acid of all at that temperature.

A fourth gas-storage experiment was started September 21, 1933. The grapefruit came from one of a group of selected trees near Corona. The arrangement of the different lots was the same as in the third experiment, except that there were nine chambers and 25-percent ethyl alcohol replaced the ammonia solution in the three corresponding lots.

On September 26 the fruit in the acidified lots at all temperatures had pitted, slightly at 32° F. but severely at the other temperatures. The acidified fruit was then removed from the three chambers, the acid solution replaced with ammonia solution (0.5 ml of 28-percent  $\text{NH}_3$  solution in 2.5 l. of water), and fresh grapefruit was added. Final inspection was made November 16. It should be noted that the ammonified lots were in storage 5 days less than the others. The results of the inspection placed the lots in the following order as regards increasing amounts of pitting and spotting and other storage blemishes: 52°, check; 52°, ammonified; 42°, alcohol; and 42°, ammonified. The check and the lot over alcohol at 52° were in perfectly sound condition and the 42° ammonified lot was rather severely pitted and spotted.

The results of the foregoing experiments on grapefruit in atmospheres to which considerable quantities of certain substances had been added indicate that acetic acid accelerated and enhanced pitting or spotting, and that alcohol and very dilute ammonia retarded it. Alcohol seemed also to have retarded the development of yellow color in storage. The experiments were not extensive enough to allow a determination of optimum concentration of ammonia or alcohol, and further work on the problem might be justified.

Nelson (19) reported the results of experiments in which he enclosed oranges and grapefruit in jars with various organic chemicals, including a number of aldehydes, esters, and alcohols. In other experiments by various means he excluded oxygen from the fruit. Many of these treatments produced injuries to the rinds that resembled closely those which appear commonly in storage. The results of such tests and of those reported in this paper have no necessary connection with storage pitting and spotting. They do, however, demonstrate that if a cell, or an island of cells, of citrus rind is injured by almost any means, the manifestations of that injury are likely to resemble the blemishes which occur "naturally" in storage. The writers, in an



experiment with Valencia oranges, used 15 organic chemicals of the anesthetic type. The apparently characteristic types of storage injuries frequently occurred in the various lots, and in several instances (e. g., with ethyl chloride, ammonium thiocyanate, and ether) single lots exhibited almost the entire gamut of the common types of storage injuries.

#### PERMEABILITY

It seemed desirable to secure some idea of the relative permeability of grapefruit-rind tissue as affected by different temperatures and treatments. Some simple experiments were accordingly conducted. These were not designed to be critical permeability experiments, and perhaps the word "permeability" is inappropriate; the results obtained, however, seemed to indicate some definite effects of temperature on the integrity of rind tissue.

The first permeability study was made September 19, 1933, the fruit used being representative of each lot from the third gas-storage experiment after the final inspection. The stem-end rinds were taken for pH determinations and the blossom-end rinds for the permeability test. The albedo and flavedo of each storage lot were separated from each other, 75-g samples of each were weighed out, and then recombined. The resulting 150-g lots of fresh tissue were rinsed with distilled water and transferred to 1-liter Erlenmeyer flasks, and 750 ml of distilled water was added. The flasks were then placed upon a turntable which in revolving gently jolted and stirred the samples. The average temperature during the period of the experiment was about 70° F. After 24 hours the water was poured off through cheesecloth into 1-liter volumetric flasks and made up to volume. Then 900 ml from each flask were transferred to beakers and placed on a water bath to reduce the volume of the sample. Later these samples were transferred to evaporating dishes to complete evaporation and for final weighing. The flavedo and albedo tissues remaining in the flasks were discarded. After the weights of the solids which had exosmosed from each lot of tissue had been obtained, the solids were redissolved in water and made up to 1 liter for the determination of reducing sugar.

TABLE 9.—*Total solids and reducing sugars exosmosed from 150 g of living Marsh grapefruit rind after storage at different temperatures and atmospheres*

Storage temperature (° F.)	Treatment	Total solids	Reducing sugar	Storage temperature (° F.)	Treatment	Total solids	Reducing sugar
		<i>Grams</i>	<i>Grams</i>			<i>Grams</i>	<i>Grams</i>
52-----	Normal-----	11.33	1.96	42-----	Acidic-----	11.37	1.86
	Basic-----	10.95	1.65		Alcoholic-----	11.85	1.71
	Acidic-----	10.62	1.68	32-----	Normal-----	12.74	1.96
42-----	Normal-----	12.27	2.31		Basic-----	11.95	1.47
	Basic-----	11.53	1.89		Acidic-----	11.82	1.56

The results of the two operations described above are presented in table 9. The rinds from the 42° F. storage appeared to be the most permeable to reducing sugar, but the amount of total solids exosmosed was greatest from the 32° and least from the 52° storage tissue. The check lots seemed to have been most permeable to both reducing sugar and total soluble solids at all temperatures. The relative

positions of the various lots at each temperature do not seem definite enough for comment. This permeability test dealt with more factors than is usual in such experiments, particularly since the lots used had been held at various temperatures, so that there were differences among them in chemical composition aside from any permeability differences that may have been caused by differences in temperature.

A second permeability experiment was started October 27, 1933, with grapefruit from Corona and Fontana, which was picked on September 12 and 14, respectively. In the intervening time this fruit had been stored at 52°, 42°, and 32° F. The stem-end rinds only were used. The flavedo and albedo were separated, and 150 g of each constituted the samples, of which there were 12 (2 localities, 3 temperatures, and 2 portions of the rind). The samples were rinsed with distilled water. They were run on a turntable continuously for 68 hours at an average temperature of about 70°. After the jolting and stirring the solutions were filtered off. The amount of solution recovered from the tissues varied with the sample. The amount of water required to make the filtrates up to their original volume was always greater for the albedo than for the flavedo tissue, greatest for the 52° tissue and least for the 32°, and greater for the Corona than for the Fontana rinds.

The filtrates were evaporated to dryness and the amount of total soluble solids determined. The results are presented in table 10. It will be seen that the apparent permeability of the flavedo was greater than that of the albedo. It may have been, however, that the greater movement of water into the albedo tissue during the experiment tended to decrease the rate of diffusion of the solutes outward. The permeability, as regards total soluble solids, was again greatest at 32° F. and least at 52° for all situations except possibly one in the Fontana albedo.

TABLE 10.—*Total solids exosmosed by the flavedo and the albedo of 150 g of living Marsh grapefruit rinds after storage at different temperatures*

Storage temperature (° F.)	Corona		Fontana	
	Flavedo	Albedo	Flavedo	Albedo
	Grams	Grams	Grams	Grams
52.....	6.50	5.30	7.47	5.59
42.....	6.98	5.68	8.28	5.52
32.....	7.26	6.61	9.78	6.99

The third permeability experiment was started January 18, 1934, with grapefruit from Oasis, which was picked December 11, 1933, and placed in storage at 52°, 42°, and 32° F., where it remained until January 17. The rinds of the three storage lots were separated into stem-end flavedo, blossom-end flavedo, stem-end albedo, and blossom-end albedo. This division gave a total of 12 different samples of 150 g each. The samples were rinsed in water, transferred to 1-liter Erlenmeyer flasks, and 650 ml of distilled water was added. The flasks were then taken to a storage room in which the average temperature was about 34°. Into each flask was placed a glass tube which reached to the bottom, while the other end was connected by rubber tubing to a manifold having 12 outlets. The manifold was then

connected through a pressure-reducing valve to one of the compressed-air lines of the ice plant. Air was released from each outlet at the rate of about 5 l per hour. The bubbles of air rising through the tissue and solution in the flasks kept the samples both aerated and stirred. The decrease in volume of solution in the flasks, due to evaporation and absorption by the tissue, was made up to mark from time to time by additions of distilled water.

The experiment ran continuously until January 28, when the flasks were brought into the laboratory. The solutions were filtered off into 1-liter volumetric flasks and made up to volume. A 100-ml portion was taken from each flask for determination of reducing sugar and the remainder was used for total solids.

When the rind portions were removed from the flasks after the solutions had been poured off, they were moist-dried between filter paper and reweighed. The gain in weight of the tissue over the original 150 g plus the weight of the total solids exosmosed gave the amount of water absorbed by each lot during the experiment.

The results of the third permeability experiment are presented in table 11. The data show that the loss of reducing sugar and total soluble solids increased as the storage temperature was lowered. The amount of water absorbed by the flavedo tissue was in the same direction, but even more strongly marked. The absorption of water by the albedo did not vary much with the previous temperature treatment, although the total amount was nearly 10 times that absorbed by the flavedo. There seemed to be a somewhat greater permeability shown by the blossom-end flavedo and the stem-end albedo. Klotz and Haas (16) found a greater general permeability at the stem end of grapefruit. It has been shown that at the close of the storage period of the fruit used in these permeability tests the reducing sugar was considerably more abundant in the rinds held at 52° and 42° F. than in those held at 32°. Yet these last rinds subsequently lost more reducing sugar during the permeability tests than the 42° and 52° lots, thus indicating even more strongly that the greater permeability indicated at 32° was real.

TABLE 11.—*Total solids and reducing sugar exosmosed from, and amount of water absorbed by, stem- and blossom-end flavedo and albedo of 150 g of living Marsh grapefruit rinds after storage at different temperatures*

End	Storage temperature	Reducing sugar from—		Total solids from—		Water absorbed by—	
		Flavedo	Albedo	Flavedo	Albedo	Flavedo	Albedo
	° F.	Grams	Grams	Grams	Grams	Grams	Grams
Stem.....	52	1.29	1.13	10.70	9.20	28.9	171
Blossom.....		1.37	1.13	11.27	9.20	28.5	178
Average.....		1.33	1.13	10.99	9.20	28.7	175
Stem.....	42	1.45	1.45	10.72	10.72	30.0	167
Blossom.....		1.70	1.33	12.27	9.78	31.6	196
Average.....		1.70	1.39	12.27	10.25	30.8	181
Stem.....	32	1.85	1.67	14.37	12.10	35.7	170
Blossom.....		1.95	1.54	14.55	9.91	33.1	184
Average.....		1.90	1.61	14.46	11.01	34.4	177

From these experiments as a whole, the conclusion is drawn that the permeability of the cells of grapefruit rinds to their own soluble constituents increased as the storage temperature was lowered. These findings are in agreement with those reported by Pantanelli (20) for endocarp tissue of the mandarin orange, although he worked at considerably lower temperatures. The increased permeability with the lowering of storage temperature might easily have an important bearing on the breaking down of fruit tissue in cold storage. The resulting increased ease of migration of certain harmful byproducts of metabolism through the tissue could allow the initiation of abnormal and injurious reactions.

Earlier in this paper the Corona-Fontana and the Oasis rinds were compared in respect to the behavior of naringin in storage. In the former rinds naringin was hydrolyzed, and in the latter synthesized, in storage. Both the hydrolysis and the synthesis were greatest at 32° F. The reasons for this latter situation are not apparent. It is possible, however, that the increased permeability found at the lower temperatures might have been partly responsible for it by permitting a more intimate association between the particular glucosidase and naringin. The increased permeability might thus offset the normal lowering of the activity of the enzyme, and so cause at 32° a more rapid hydrolysis or synthesis of naringin as the balance favored one or the other reaction.

#### CONDUCTIVITY

When it was learned that the permeability of grapefruit rinds increased as the storage temperature was lowered it seemed desirable to determine what changes might take place in the conductivity of rinds under similar circumstances. Accordingly, a number of tests were carried out with grapefruit rinds from storage samples and from samples picked for immediate study. The instrument used was a Leeds and Northrup student potentiometer equipped for conductivity measurements, consisting of a microphon hummer, telephone receivers, and a conductivity cell of the dip type.

The first attempts were directed toward the measurement of the conductivity of the rinds in situ, but the results were so extremely variable that the method was abandoned. Subsequently the measurements were made on juice expressed from the rinds by using the dip type conductivity cell previously mentioned. The variability of the results was still too great to allow any conclusions except of the most general sort. The total range of resistance of the rind juices, as recorded from about 200 determinations, made with the same cell at 22° C., was 150 to 515 ohms. The range in any one storage series was usually less than 50 ohms. The trends shown by the data are presented below.

(1) The earlier determinations seemed to show that the lower the storage temperature, the greater was the conductivity, but so many exceptions were later found that this conclusion did not appear to be justified.

(2) The difference in conductivity between stem and blossom-end rinds was very slight. The total average resistance of all such determinations of the rinds of field samples taken for immediate study gave 339 and 330 ohms for the stem and blossom ends, respectively. Thus, there was a doubtful tendency toward higher conductivity at

the blossom end. When the results of corresponding determinations on the storage samples were examined, the situation regarding stem and blossom ends was found to be reversed; that is to say, the stem-end rinds showed the higher conductivity.

(3) In all instances recorded the flavedo showed a higher conductivity than the albedo, but the difference was less in the storage samples. This situation may help to explain the apparent reversal of conductivity of the stem- and blossom-end rinds in storage, since the stem-end rind had a larger proportion of albedo.

#### PECTIN DETERMINATIONS

In order to determine whether there is any relation between grapefruit spotting and the condition of the pectic substances of the rind, a preliminary set of determinations was made on fruit which had been held in storage.

The method used was developed from that given by Carré (3), and Carré and Haynes (4), and modified by Haller (10), Rosa (21), and Conrad (6).

Fruit was collected on June 15, 1934, from previously selected trees and divided into three equal lots for storage at 36°, 42°, and 52° F. It was removed on July 24 for the determination of pectic substances. At that time all the fruit at 36° was severely spotted; all at 42° was slightly spotted; and all at 52° was sound. The pectic contents of the stem-end rind are given in table 12.

TABLE 12.—*Pectic content of the Flavedo and albedo of rinds of Marsh grapefruit grown at Claremont, Calif., and stored 39 days at different temperatures*

[Figures are in percentage calculated as calcium pectate on fresh-weight basis]

Substance determined	36° F.		42° F.		52° F.	
	Flavedo	Albedo	Flavedo	Albedo	Flavedo	Albedo
Soluble pectic acid.....Percent..	0.0	0.0	0.0	0.0	0.0	0.0
Soluble pectin.....do.....	.24	.72	.40	.84	.28	.68
Insoluble pectic acid.....do.....	0	0	0	0	0	0
Protopectin.....do.....	2.31	1.71	2.56	1.90	2.44	2.01
Total.....do.....	2.55	2.43	2.96	2.74	2.72	2.69
Protopectin: pectin.....ratio..	9.8	2.4	6.4	2.3	8.7	3.0

Soluble pectin was always higher in the albedo, and protopectin in the flavedo, the total pectic content being slightly higher in the flavedo. No pectic acid, either soluble or insoluble, was found in any sample. The ratio of protopectin to pectin was always higher in the flavedo than in the albedo, the average being 8.2 and 2.6, respectively.

There was no correlation between pectin content and temperature, on the one hand, and spotting of the fruit on the other. However, the soluble pectin content of the fruit held at 42° F. was higher, both in the flavedo and the albedo, than in that held at the other temperatures; this brought about a lowering of the ratio of protopectin to pectin (in the combined flavedo and albedo) to an average of 4.4 at 42° as compared with 6 and 5.9 at 36° and 52°, respectively. The

most serious spotting has generally occurred at 42°. More work needs to be done to determine whether there is any definite relationship between spotting and the condition of the pectic contents of the rind.

#### BUFFER SYSTEM

The frequent tendency of the rind of grapefruit stored at 42° F. to be higher in active acidity than that stored at a higher or lower temperature suggested the desirability of determining whether the different temperatures had different effects on the buffer system.

The fruit used for this experiment was collected at Claremont on June 15, 1934, and various lots were stored at 36°, 42°, and 52° F. At the end of 5 weeks all the fruit at 36° was severely spotted, that at 42° was slightly spotted, and that at 52° was sound. Only the stem-end rind was used.

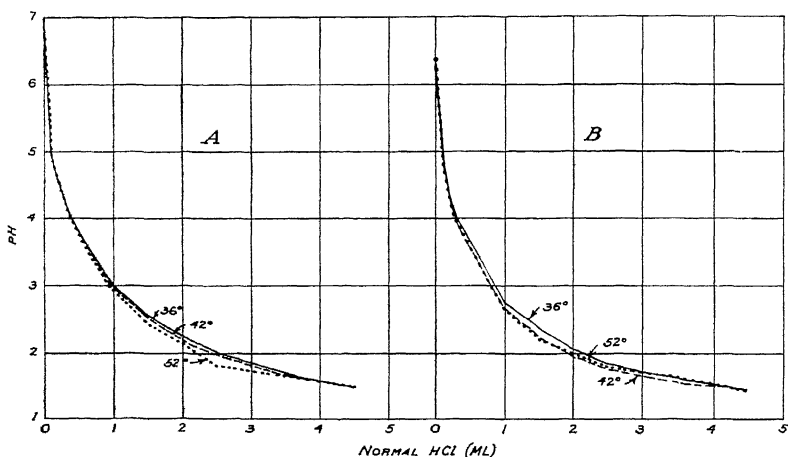


FIGURE 13.—pH of the flavedo (A) and the albedo (B) of the rinds of Marsh grapefruit, from Claremont, Calif., after 5 weeks' storage at 36°, 42°, and 52° F., after addition of various quantities of normal HCl. Ten grams of freshly ground tissue in 50 ml of water was titrated with normal HCl as indicated.

The method used was as follows: The fruit was peeled and the flavedo and albedo were separated. The rind was ground in a food chopper equipped with a nut-butter attachment. A 10-g sample was weighed out and 50 ml of water added and the mixture stirred well. Quinhydrone was then added and the mixture stirred again by means of a mechanical stirrer for 1.5 minutes before the initial reading on the potentiometer was made. The stirring apparatus consisted of two parts, one a glass paddle immersed in the liquid and constantly turning and the other a small turntable upon which the beaker was placed. In this manner the entire contents of the beaker were kept in continuous motion while the acid or base was being added and the readings were being made. The paddle and turntable were turned by means of a small electric motor. The high viscosity of the material used necessitated such an arrangement in order to get a uniform mixing of the acid or base after each addition.

Immediately after the first reading was taken 0.1 ml of normal acid or base was added. The whole was allowed to mix 1.5 minutes

before determining the resulting pH. This procedure was repeated with the base till a pH value of about 7 was attained. When acid was used, 0.1-ml portions were added till 0.5 ml had been added, after which 0.5-ml portions were added and later 1.0-ml portions, until about pH 1.5 was reached.

In order to attain pH 1.5 it was necessary to add about 4.5 ml of normal hydrochloric acid to both the flavedo and albedo from all the temperatures. After this amount of acid had been added the pH of the flavedo was 1.50 in rind from the 36° F. storage, 1.51 in that from the 42°, and 1.50 in that from the 52°. The corresponding pH values of the albedo were 1.44, 1.43, and 1.42, respectively. This shows no change in the buffer system brought about by differences in storage temperatures. The titration curves are given in figure 13.

A rapid drop in pH occurred on the addition of the first few tenths of a milliliter of normal acid. In order to see whether there might be differences in this range too small to be observed with the concentration of acid used, another series of titrations was carried through. One-tenth normal hydrochloric acid was used and the same quantities per reading were added as before. Only the albedo was tested. No important differences were observed between the rinds held at the different temperatures. During the addition of the first 0.6 ml the pH of the three rinds dropped at the same rate (fig. 14).

From then until 1 ml had been added the drop in the pH of the rind stored at 36° F. was most rapid, next in that held at 52°, and smallest in that held at 42°, indicating the strongest buffer in the material from 42°. At this time the pH attained was 4.78, 4.85, and 4.93, respectively. These relative positions were maintained with gradually diminishing differences until 10 ml had been added, when the pH values were 2.91, 2.91, and 2.94, respectively. At no time was there any indication of a destruction of any part of the buffer system during storage at a temperature of 42°. The titration curves with 0.1 normal acid are shown in figure 14.

A repetition of this experiment with fruit from Corona and Fontana held at the same temperatures gave similar results. However, with the Corona and Fontana fruit an average of 3.8 ml normal acid was required to bring 10 g of the albedo to pH 1.5, and 5.6 ml for the flavedo. This compares with 4.5 ml for each flavedo and albedo sample from the Claremont fruit.

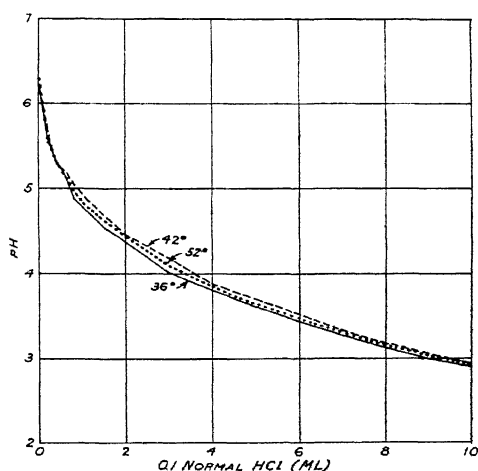


FIGURE 14.—pH of the albedo of the rinds of Marsh grapefruit from Claremont, Calif., after 5 weeks' storage at 36°, 42°, and 52° F., after addition of various quantities of N/10 HCl. Ten grams of freshly ground tissue in 50 ml of water was titrated with N/10 HCl as indicated.

## INJECTION EXPERIMENTS

## GENERAL

Tests were made to determine the effects of certain substances, principally naringin and naringenin, when injected into living grapefruit rinds.

On October 23, 1933, a few grapefruit were set aside from a storage lot which was being preserved for chemical analysis. A 0.5-percent solution of naringenin, the phenolic derivative of naringin, was made up in 5-percent alcohol. Each grapefruit was given three injections of 1 ml of this solution by means of a hypodermic needle thrust diagonally beneath the flavedo layer. The injected fruit was left in the laboratory for 24 hours. On dissection, some of the fruit showed a very slight discoloration in the vicinity of the injection. The remainder of the fruit was taken to a cold room and left at  $-15^{\circ}$  F. for 24 hours, and then brought back into the laboratory and thawed rapidly. When the injected regions were cut across, a pink color was noticed. From time to time it had been noted that the albedo of fruit which had been held in storage at  $42^{\circ}$  often showed considerable pink color after being frozen in the hardening room in preparation for pH determinations. This pink color had not been noticed at  $32^{\circ}$  or  $52^{\circ}$  and its appearance now in association with naringenin was considered very interesting.

In a second injection experiment the following solutions were used: (1) 1-percent solution of naringin in 5-percent alcohol; (2) 1-percent solution of naringenin in 5-percent alcohol; and (3) 5 percent of alcohol in water. The fruit was from Corona and Fontana and had been kept for 4 weeks at  $52^{\circ}$ ,  $42^{\circ}$ , and  $32^{\circ}$  F. There were four fruits of each lot and each fruit was injected with 1 ml of each of the three solutions named. After injection they were left in the laboratory for 40 hours and then frozen for 24 hours at  $-15^{\circ}$ .

An examination of the fruits after they had thawed showed no coloration around any of the injections in the  $32^{\circ}$  F. lots. All of the  $42^{\circ}$  lots showed a pink color around the naringenin injection. At  $52^{\circ}$  the Fontana lot showed one case of pink color for the naringenin solution. No other solutions produced the pink color.

On November 20, 1933, 27 grapefruit freshly picked at Oasis were injected and stored at  $32^{\circ}$ ,  $42^{\circ}$ , and  $52^{\circ}$  F. for 12 days. The solutions used were 0.1 percent of naringin, 0.1 percent of naringenin in 5-percent alcohol, and 5-percent of alcohol as a control. Injury was evident earlier in the lots kept at  $32^{\circ}$  and  $42^{\circ}$ . By the close of the period all naringenin injections at all temperatures had caused serious injury in the form of discolored depressions 1 to 2.5 cm across. There were four traces of injury from 27 injections of the naringin solution but none with the control 5-percent alcohol solution. In general, the injuries were most severe at  $42^{\circ}$ .

A fourth injection experiment was started on January 20, 1934, with fruit that had been freshly picked at Claremont. Each fruit was injected with 2 ml of each of five solutions, as follows: (1) Control, 5-percent of alcohol; (2) 0.1 percent of naringin in 5-percent alcohol; (3) 0.1 percent of naringenin in 5-percent alcohol; (4) 0.028 percent of naringenin in distilled water; and (5) ferric chloride solution (2 drops of 50-percent solution to 100 ml of water).



Within 4 days the fruits injected with solution no. 3 showed injury at all three storage temperatures and solution no. 5 had caused dark depressions. An inspection on January 29 gave the following results: For solution no. 1, no injury evident; solution no. 2, no injury (all injections appeared exactly like the controls); solution no. 3, depressions and other evidences of injury around every injection (depressions smaller at 32° F.); solution no. 4, some injury, particularly lack of firmness around injections; and solution no. 5, darkening present in all cases. The darkening seemed to be mostly the characteristic reaction of ferric chloride with the naringin occurring naturally within the tissue.

The appearance of the injected areas is shown in plate 2, *A*, *B*. *A* is a photograph of two fruits taken at random from the injected lots shortly after inspection and removal from storage. The 0.1-percent naringenin injections are facing forward in each case. The 0.1-percent naringenin injections are to the left, and the 0.028-percent naringenin injections are to the right. The crayon circles on the fruits were in different colors to aid identification of solutions. Plate 2, *B*, is a photograph of the same fruit shown in *A* but taken after the fruit had remained at room temperature for 24 hours. The photograph shows how the naringenin depressions had darkened and become more severe.

A fifth injection experiment was planned to test further the effects of naringenin solutions of various strengths. The fruit used was from Claremont and had been in storage at 32°, 42°, and 52° F. for 26 days. Five fruits were selected for each experiment, and each fruit was injected with 2 ml of the following solutions: (1) Check, 5-percent alcohol; (2) 0.1 percent of naringenin in 5-percent alcohol; (3) 0.05 percent of naringenin in 5-percent alcohol; (4) 0.01 percent of naringenin in 5-percent alcohol; (5) 0.005 percent of naringenin in 5-percent alcohol. After injection the fruit was returned to the respective storage rooms. Inspection in 2 weeks gave the following results: At 32° all five fruits injected with solution no. 3 showed sunken and darkened areas and two injected with solution no. 4 also were injured. All others were sound. Numerically the effects were the same at 42° and 52°, but the degree of injury was greatest at 32° and about the same at 42° and 52°.

A sixth experimental injection was made on fruit selected from the same trees as for the fifth experiment. In the sixth experiment, however, the fruit was injected on the day of picking instead of after a period in storage. The injections were like those in the fifth experiment, and after injection the fruits were placed at the three temperatures previously used. Inspection was made after 2 weeks. At 32° F. all five fruits injected with solution no. 3 were badly affected, as were two of those injected with solution no. 4. No others were affected. At 52° there was no injury from solution no. 4. At 42° the results were numerically the same as at 32°. The relative severity of injury at the different temperatures was in decreasing order at 42°, 32°, and 52°.

#### SPECIFIC TESTS

In the injection experiments described above no attempt was made to keep the fruit from the different trees separate. The fruit from each picking was placed in one general lot before injection, the divi-

sion being made later for storage at different temperatures. It was noted that certain fruit in the injected lots escaped, or almost escaped, being affected by the naringenin in solutions. Because of the possibility that these manifest differences in susceptibility might be partly the result of differences in individual trees, all further injection tests were made with fruit of definitely known origin.

On February 16, 1934, 15 fruits from each of the 3 trees at Claremont were injected with 0.05-percent, 0.025-percent, and 0.01-percent naringenin solutions. After injection the lots were separated and placed at the three usual storage temperatures. In 4 days inspection indicated (1) that injury was most abundant and severe at 42° F., and (2) that the fruit from tree no. 2 was least injured and that from tree no. 3 most injured.

On February 27 fruit was collected from the same three trees at Claremont. One set was used for the injection test and another for storage at the three temperatures. The injection solutions were 0.03 and 0.015 percent of naringenin. After 5 days' storage, inspection showed that the injuries around the injections were most severe at 42° F. and that the fruit from tree no. 2 showed least and that from tree no. 3 most injury.

The uninjected lots which had been placed in storage were inspected for storage spots about 5 weeks later. There was only a trace of spotting at 52° F., but all fruits at 42° and 32° were slightly spotted. The least severe spotting at the last temperatures occurred on the fruit from tree no. 2.

These injection and storage tests were repeated on fruit from the same trees four more times, on March 19, March 31, May 5, and May 18, and each injected lot was held for 5 days at 42° F. The injection tests in every case showed least injury to fruit from tree no. 2 and most injury to fruit from tree no. 3. In the storage tests the fruit from tree no. 2 always showed the least severe spotting, but that from trees nos. 1 and 3 alternated their order in being most severely affected.

In the tests started March 19 and March 31, fruit from 7 additional trees was collected and injected, together with that from the original 3 trees, and held for 5 days at 42° F. In the first test with fruit from these 10 trees, the three lots least affected by the injections were from trees 2, 4, and 6; those most affected were from trees 9, 8, and 10. In the second test the lots least affected were from trees 4, 5, and 6, and those most affected were from trees 9, 1, and 10.

Similar injection experiments were conducted at about the same time with fruit from selected trees at Oasis. The results need not be described in detail, since they were in general similar to those secured from the experiments with fruit from Claremont. Both series of tests showed that susceptibility to injury from injections of naringenin varied from tree to tree, but persisted in the same relative degree in fruit from the same tree throughout the period of the tests.

#### DISCUSSION

The results of all the injection tests with grapefruit show that naringenin is extremely toxic to rind tissue. Except in the early tests the strongest solution used was 0.03 percent, but even the 0.015-percent solution was much stronger than was necessary for

the most susceptible fruit. When the 0.015-percent solution was used, each injection consisted of only 0.3 mg of naringenin. This amount when injected into the albedo produced sunken and discolored areas 2 cm or more in diameter. Slight injury was caused by 0.05 mg of naringenin. On the other hand, naringin was only slightly toxic to rind tissue. Thirty to fifty milligrams of naringin have been injected into grapefruit rinds without injuring them. The injuries caused by naringenin are frequently very similar to the common storage spot, both in shape and color, as plate 2, *C*, shows. These fruits had been injected with 0.6 and 0.3 mg of naringenin 2 weeks earlier and held at 42° F. Discolored spots were produced by both quantities of naringenin.

The conclusion is not drawn, from the effect of naringenin on rinds recorded above, that this substance must be the principal cause of certain storage spots; it does seem very probable, however, that grave injury to the rind would result from any metabolic disturbance that would either retard the synthesis of naringin from any naringenin present or hinder the rapid disposal of this derivative as freed by hydrolysis. Zoller (24), in his study of naringin, pointed out the possible danger of naringenin to the tissue and suggested that certain pink spots might have been caused by it. In the present experiments it has been shown that naringenin is not only very effective in producing the more common sunken and discolored spots, but also that it produces a pink color in the albedo, which appears to be identical with that developing in uninjected rinds held at 42° F. and subsequently frozen.

In general, the naringenin injuries were most severe when the fruit was held at 42° F. for a few days after injection. It is an interesting fact that naringenin was most effective in causing spotting at temperatures that give the greatest amount of common storage spotting. If an extremely delicate and specific microchemical test for naringenin were available some relation between this substance and storage spotting might be established. So far all the microchemical reactions of naringin and naringenin have been found to be the same.

Other considerations arise from the results of the specific injection experiment in which the fruit of each tree was treated separately. There was a difference in susceptibility to naringenin injury in different fruits and there also seemed to be some consistency in the apparent susceptibility of fruit from different trees. In some instances this susceptibility to naringenin injury seemed to be correlated with the amount of storage spotting of fruit from the same trees. The susceptibility to naringenin injury seemed to vary with the stage of maturity in somewhat the same way as the tendency to spot in storage.

#### SUMMARY

Results are presented of two series of studies of the changes that take place in the rind of Marsh grapefruit, one in the field, the other in storage. One of the field studies was carried out during the summer of 1933 in the general district that includes Fontana and Corona, Calif., and the other during the winter of 1933-34 in a single locality near Oasis, Calif. These localities represent two important climatic districts concerned in the production of grapefruit.

Seasonal changes in the chemical composition of the rind from the three localities were as follows: (1) Water content showed a slight seasonal trend upward in all three localities. (2) The flavedo-albedo ratio at Fontana and Corona increased slowly to July 23 and then fell below the values found at the beginning of the season. The average ratios were 0.71 and 0.77, respectively. At Oasis the ratio fluctuated with weather conditions, the average being 0.81. (3) Soluble solids increased throughout the season in all three localities, except for a slight falling off at the last collection at Fontana and Corona. (4) Total sugar at Fontana and Corona increased steadily to July 23, then decreased; the graphs for total sugar paralleled those for mean air temperature. At Oasis total sugar tended to increase through the season. (5) Invert sugar at Fontana and Corona increased to July 23, then decreased. At Oasis it did not change significantly until after the third collection, when it increased. The invert-sugar graph paralleled the mean air-temperature graph at Oasis and at Fontana and Corona. (6) The graphs for reducing sugar at Fontana, Corona, and Oasis were somewhat the inverse of the mean-temperature graphs. (7) Naringin decreased in general through the season at all localities, but there were fluctuations due to local conditions. (8) Hydrolyzable polysaccharides showed a steady relative decrease through the season at all localities. (9) Hydrogen-ion concentration of the rind showed only a slight seasonal drop at all localities and that of the pulp juice changed still less.

When the stem-end and blossom-end rinds were compared the following differences were found: (1) At Fontana and Corona the stem-end rind had more water, reducing sugar, invert sugar, and soluble solids than did the blossom end. The latter was higher in hydrolyzable polysaccharides and hydrogen-ion concentration. Naringin and the flavedo-albedo ratio were practically the same in both ends. (2) At Oasis the average differences between the stem ends and blossom ends were very slight except in invert sugar, in which the blossom-end value was significantly higher.

In a comparison of the flavedo and albedo it was shown that (1) the differences between flavedo and albedo tissues were smaller in the grapefruit rind than in the orange rind; (2) the differences were smaller at Fontana and Corona than at Oasis; and (3) the flavedo was higher than the albedo in invert sugar, total sugar, and soluble solids, whereas the albedo was higher in naringin, hydrolyzable polysaccharides, and hydrogen-ion concentration.

The Fontana and Corona rinds agreed more closely in composition than either agreed with the Oasis rinds. The Corona rinds showed a somewhat closer resemblance to the Oasis rinds than did the Fontana rinds.

The main storage experiments were conducted on fruit from Corona, Fontana, and Oasis, but the results of a number of miscellaneous storage experiments on grapefruit rinds are also presented.

A study of pitting and spotting in relation to storage showed:

(1) Fruit from all three localities pitted and spotted most severely at 42° F. and least at 52°, with 32° intermediate.

(2) Mature fruit picked at Corona and Fontana early in the harvest period was more severely affected at 42° F. than that picked in the latter part of the harvest period; at 32° this pitting and spotting

was the reverse of that at 42°; at 52° there was never more than a trace of storage blemishes.

(3) The fruit from Oasis varied strikingly with the season in its susceptibility to pitting and spotting, when held at 32° and 42° F. No pitting or spotting occurred at 52°; but at 32° it increased as the season advanced. At 42° pitting and spotting reached their maximum early in the season, dropped to a minimum in midseason, and increased again toward the end of the harvest season.

Chemical analyses of the rinds of Corona and Fontana grapefruit showed:

- (1) Water content decreased slightly at all temperatures.
- (2) Total sugar decreased at all temperatures but least at 32° F. and most at 52°.
- (3) Reducing sugar decreased slightly at 32° F. but increased at the higher temperatures.
- (4) Invert sugar decreased at all temperatures. The decrease was slight at 32° F., but striking and about the same at 42° and 52°.
- (5) Naringin usually decreased. The decrease was greatest at 32° F. and least at 52°.
- (6) Hydrogen-ion concentration increased at all temperatures. The increase was greatest at 42° F.
- (7) There was a high positive correlation between hydrogen-ion concentration and naringin content.
- (8) Hydrolyzable polysaccharides increased at all temperatures.
- (9) Soluble solids decreased slightly at all temperatures.
- (10) Rinds of the fruit from Corona and Fontana behaved essentially alike in all the reactions noted.

Chemical analyses of the rinds of the Oasis fruit showed:

- (1) A slight increase in water at 52° F. and a slight decrease at 42° and 32°.
- (2) Total sugar decreased at 42° F. and 52°, but increased slightly at 32°.
- (3) Reducing sugar increased slightly at all temperatures.
- (4) Invert sugar behaved almost exactly as in the Corona and Fontana rinds in all storage collections.
- (5) Hydrogen-ion concentration increased in storage at all temperatures but least at 32° F.
- (6) Naringin increased at all temperatures, most at 32° F. and least at 52°. The total naringin content showed a high positive correlation with hydrogen-ion concentration.
- (7) Hydrolyzable polysaccharides and soluble solids changed only slightly.

A comparison of the Oasis and Corona-Fontana grapefruit rinds showed:

- (1) Differences in prestorage composition.
- (2) Naringin was hydrolyzed in storage in the Corona-Fontana rinds and synthesized in the Oasis rinds.

Miscellaneous storage experiments showed:

- (1) Fruit held for one period at 52° F. and then transferred to lower temperatures spotted only slightly; fruit held at either 42° or 32° continued to spot when transferred to 52°.
- (2) Weakly acidified atmospheres accelerated and enhanced pitting and spotting, whereas weak alcohol and ammonia seemed to retard it.

Alcohol also retarded the development of yellow color in the fruit in storage.

(3) The permeability of grapefruit rinds to their own soluble substances increased as the storage temperature was lowered.

(4) The conductivity of the flavedo was higher than that of the albedo, but this difference was reduced during storage. The difference between stem and blossom ends was slight. Variability of conductivity in the same lots of tissue was relatively great.

(5) Soluble pectin was higher in the albedo, and protopectin and total pectic substances were higher in the flavedo. No pectic acid was found in any instance. The soluble pectin content was highest at 42° F. and the protopectin-pectin ratio lowest.

(6) No significant alteration of the buffer system due to storage temperatures was noted.

Injection experiments showed:

(1) When solutions of naringin and its phenolic derivative, naringenin, were injected into the albedo of grapefruit rinds naringenin was approximately 1,000 times more toxic to rind tissue than naringin.

(2) After rinds were injected with 0.1 to 0.6 mg of naringenin and held for 5 days at 42° F., they often showed spots similar to those commonly observed in storage.

(3) If rinds were injected with naringenin and held for a few days at 42° F., then frozen and thawed, the injected areas showed a pink coloration similar to that sometimes observed in stored grapefruit. In rinds injected with dilute naringenin solutions the injury was most aggravated by holding at 42°, which, of the three temperatures used throughout the storage experiments, caused the greatest amount of spotting.

(4) Individual fruits showed different degrees of susceptibility to naringenin injury. Furthermore, the fruit of individual trees seemed to show collectively somewhat consistent relative susceptibility or immunity to injury from given quantities of naringenin. Usually, uninjected fruit from the extreme-case trees mentioned above, when stored for several weeks at 42° F., developed degrees of storage spotting in rather interesting agreement with the susceptibility or immunity to naringenin injury previously noted for them.

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# THE HONEYBEE AND THE BEEHIVE IN RELATION TO FIRE BLIGHT<sup>1</sup>

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## INTRODUCTION

The chief objective in fire blight control has been the eradication of hold-over cankers, since these cankers are known to be the principal source of primary inoculum in the spring. Consequently the possibility suggested by Rosen (11, 12)<sup>3</sup> that the fire blight organism, *Erwinia amylovora* (Burr.) Bergey et al., may overwinter in the beehive has complicated the problem by introducing one more possible source of infection. Following the appearance of Rosen's first report in 1930, studies were begun in several States to determine the role played by the honeybee in the dissemination of the bacteria and to ascertain whether the bacteria overwinter in the beehive.<sup>4</sup> The findings from New York, Ohio, and California detailed later, which are at variance with those from Arkansas, receive further support from this undertaking, an abstract of which has already been published (5).

The fact that the honeybee is an important pollinating agent for fruit blossoms and that individuals from colonies of bees regularly visit flowers at a time when they are highly susceptible to infection by the fire blight organism makes this problem a most important one. It must, however, be remembered that the worker bees which visit blossoms are not those which have had contact with contaminated material the year before, since the life span of the worker is short. Normally no bee would be living in the colony at the time of visits to fruit blossoms which had been there the year before except the queen bee, and queen bees do not visit flowers. There remains, however, the somewhat remote possibility that in some manner the queen bee might be involved in harboring the fire blight organism. Attention must also be directed to other places within the hive where the fire blight bacteria might remain, such as in honey or in other materials in and about the hive. These facts, then, center the investigation largely on the behavior of the bacteria when introduced into the hive, and it is on this point that special emphasis has been placed in this work.

The chief aim of this investigation has been: (1) To determine the longevity of the fire blight organism in the beehive when introduced through the natural channel in the food of the bees, but other aspects of the problem have also received attention, such as (2) the longevity of the bacteria in sugar solutions studied over a wide range of concentrations and temperatures; (3) the location of the bacteria in relation to the life-cycle stages of the insect; and (4) the extent of

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<sup>2</sup> The writers are indebted to Dr. A. W. Woodrow, formerly of the Department of Entomology, for assistance in handling the bees used in these studies.

<sup>3</sup> Reference is made by number (italic) to Literature Cited, p. 809.

<sup>4</sup> Since the environment within the beehive may be assumed to be virtually identical throughout the country the results from the several States should be in close conformity with each other.

dissemination of the bacteria from the beehive to flowers in cages in the orchard.

In the spring of 1932 during the course of observations on the conduct of honeybees visiting pear and apple flowers in the orchard of Cornell University, it was observed that some of the flowers farthest along in development presented a water-soaked appearance. Examination under the microscope showed the flower tissues to be gorged with bacteria. Noting that the bees visited flowers in the clusters containing these water-soaked individuals, a preliminary isolation experiment was made to determine whether the fire blight bacteria were associated with the bees. When the first apple petals were beginning to fall, 10 bees were captured from blossom clusters containing such water-soaked blossoms. At three subsequent biweekly intervals, when blossoms were no longer available, lots of 10 bees each were taken at the entrances to hives in an apiary approximately a half mile away. The fire blight bacteria were obtained from 3 of the 10 bees captured from the blossoms, but no organisms were recovered from the bees subsequently taken in the apiary. In the blight epiphytotic which developed that spring as many as 90 percent of the blossom clusters in Wealthy and 50 percent in McIntosh blighted, providing ideal conditions for this isolation experiment. However, because the bacteria were obtained from the bees only while they were visiting the flowers in the orchard and not thereafter, it appeared that the organisms were incapable of remaining associated with the bees after the blossom period. Accordingly experiments were planned in an attempt to clarify the situation.

#### LONGEVITY OF THE FIRE BLIGHT BACTERIA IN THE BEEHIVE PREVIOUS OBSERVATIONS

Although Waite (20, 21) was the first to implicate the honeybee with the dissemination of the fire blight organism, it remained for Gossard (2) and for Gossard and Walton (3, 4) to initiate studies on the longevity of the bacteria in the beehive. In the first report, Gossard (2) concluded that the organism of fire blight may live in honey for 47 hours. Later Gossard and Walton (3) extended the period of viability in honey to between 72 and 100 hours. In their final report (4) they stated that during the blossom period fire blight developed in tender twigs that were inoculated with honey drawn from three different hives. Blight apparently resulted also when apple pollen, removed from the pollen baskets (corbiculae) of bees and the mouth parts of similar bees caught as they were entering the hives were inserted into incisions in tender apple twigs. They further state (4, p. 86):

We were unable to obtain cultures of fire blight from hives in early spring, from either honey or wax and because of its eventual death in honey, we believe it does not exist in the hive at the opening of the season. Dozens of samples were taken and cultured by the plate method in this quest.

The investigations of Gossard and Walton have been extended by a number of investigators. Rosen (11, p. 302) stimulated interest in this question when he stated that—

\* \* \* the writer has successfully isolated the fire-blight pathogen from beehive material gathered throughout the summer, winter and early spring and from the bees themselves obtained from the hives in the early spring prior to the development of blight.

If this statement were applicable elsewhere it would mean that fire blight control cannot be successfully accomplished by canker eradication alone. Thomas (15) reported that when the organism causing fire blight was introduced into honey and applied to the surface of honeycomb and wood frame, it was recovered after 15, 55, and 20 days. In Rosen's final report (12) covering 5 years' work, he had obtained only 1 positive culture in a total of 1,277 isolation trials from 1931 to 1933, which is in strong contrast to 20 positive isolations in 2,157 trials for 1929 and 1930. The materials used consisted of honey plus comb and bees taken from beehives located in a blighted orchard. The results of a study by Thomas and Ark (17) conducted in California did not support Rosen's conclusion. In no instance did they obtain the blight organism from hive material (550 cultures), and they obtained it from bees only when blossoms of fruit trees were available to the insects. Pierstorff and Lamb (10) also were unable to demonstrate the presence of the fire blight bacteria on the combs, frames, or in the honey in a beehive 24 hours after a water suspension of the organism had been introduced. They were successful, however, in recovering bacteria from the heads of the honeybees taken from a hive 2 days after it was contaminated with the organism. Parker (9) was unable to reisolate the bacteria in the early spring from beehives that were artificially contaminated the preceding November.

#### MATERIALS AND METHODS

Any study in which honeybees are used for experimental purposes should take into account the habits and condition of the insects. Besides the fruit-blossom period in May, early October is a good time for conducting such a study since the incoming supply of nectar is low in these months and the bees are inclined to stay inside the hive and to concentrate sugar solutions with relative rapidity.

The feeding of honeybees with artificially prepared food containing an abundance of bacteria introduces the bacteria into the hive in a manner comparable to such natural channels as may exist, obviates the necessity for disturbing the working conditions of the bees, insures the entrance of bacteria in great numbers and at the same time facilitates the reisolation of the bacteria at stated intervals after their entrance. These precautions were needed since present isolation methods are not sufficiently selective to be reliable for small numbers of these bacteria. Moreover, this approach takes into account the effect on the nectar or sugar solution produced by the bees in their function of elaborating honey from nectar.

The same two colonies of bees were used throughout the series of tests, which extended over a period of four seasons. Each colony or nucleus hive was fed diluted honey or cane sugar (sucrose) solution of 60 percent sugar concentration in the autumn, or 50 percent concentration when fed in the spring, to which vast numbers of the fire blight bacteria had been added.

The sugar solutions were prepared under approximately aseptic conditions in the same way for each of the six feeding experiments involving hives of bees. Approximately 5 gallons of the prepared solutions were fed to the respective colonies in the fall and 1 quart to

the nucleus hives<sup>5</sup> used in the spring. The sucrose solution was sterilized at 15 pounds pressure for 20 minutes. The diluted honey was brought to a boil. When cool, the bacteria in water suspension were added. The individual bacteria were thoroughly distributed in suspension by careful stirring in the thinner solutions so that in subsequent concentration of these solutions by the bees, there was ample opportunity for the solutions to act in whatever manner they would on the organisms. These precautions were taken in order to obviate the possibility that groupings or clumps of the organisms may protect some bacteria from the action of the concentrated solutions (honey) and thus increase their longevity.

The bacteria for furnishing the inoculum were varied. In 1932, 1 culture from California was employed; in 1933 a composite of 30 cultures; in 1934 a composite of 4 cultures; and in 1935 the progeny of a single-cell culture from New York. The numbers of bacteria per cubic centimeter of solution fed in series 1932a and 1933a were computed by means of the Petroff-Hausser direct bacteria counter and were, respectively, 192 and 155 millions. In the other series the numbers of bacteria were made comparable by means of the McFarland nephelometer (?).

The inoculum except when stated otherwise consisted of a 2-day-old growth on nutrient agar.

The isolation method used consisted of: (1) The transfer of relatively uniform quantities of material with a 5-mm loop to nutrient-dextrose broth, and (2) the subsequent transfer to nutrient agar when growth was evident. The culture-plate method used in the preliminary studies was later abandoned. All the cultures obtained during summer, autumn, and winter that simulated the fire blight organism were inoculated into green Kieffer pear fruits. Shoots on pear and apple trees grown in the greenhouse were used for making pathogenicity tests in the spring when fruits were not available. The surface of fruits was sterilized with alcohol and after inoculation incubated in moist chambers at 24° C.

In pursuing the investigation on the longevity of the fire blight organism in the beehive, a series of six isolation experiments were conducted in October and May. These are designated 1932a, 1933s, 1933a, 1934s, 1934a, and 1935s.<sup>6</sup>

#### AUTUMN TRIALS

##### EXPERIMENT 1932a

In experiment 1932a, the fire blight bacteria disappeared within 1 week after the close of the feeding period from honey elaborated from food containing the organisms. The bacteria had likewise disappeared from (1) the wax of the combs, (2) the surface of wooden frames, and (3) the mouth parts, alimentary tracts, and honey stomachs of the bees. A summary of this experiment appears in table 1.

<sup>5</sup> "Nucleus hive" is a term employed by beekeepers for small hives used in mating queen bees. These small hives were used because the full colonies were too large to be included in the cages employed.

<sup>6</sup> The symbols s and a signify, respectively, spring and autumn trials.

TABLE 1.—Summary of isolation studies to determine the longevity of *Erwinia amylovora* in the beehive when introduced through the natural channel of foods in spring (s) and autumn (a) trials, 1932–35

Source	Time interval	1932a <sup>1</sup>		1933s		1933a		1934s		1934a		1935s	
		Trials	Positive	Trials	Positive	Trials	Positive	Trials	Positive	Trials	Positive	Trials	Positive
	Days	No	Pct.	No.	Pct.	No	Pct	No.	Pct	No	Pct	No	Pct.
Honey-----	1	-----	-----	-----	-----	40	20.0	-----	-----	30	33.3	-----	-----
	2	-----	-----	-----	-----	40	10.0	-----	-----	30	6.7	-----	-----
	3	-----	-----	-----	-----	40	.0	-----	-----	30	6.7	-----	-----
	Over 3	100	0.0	30	0.0	320	0	30	0.0	90	.0	30	0.0
Wax-----	1	-----	-----	-----	-----	20	.0	-----	-----	15	13.3	-----	-----
	Over 1	50	0	30	.0	200	.0	30	.0	70	0	30	.0
Wood-----	1	-----	-----	-----	-----	20	.0	-----	-----	15	0	-----	-----
	Over 1	50	0	30	.0	200	2.5	30	.0	70	.0	30	.0
Bees-----	1	-----	-----	-----	-----	100	6.0	-----	-----	85	11.8	-----	-----
	Over 1	50	.0	30	.0	50	.0	30	.0	50	.0	30	.0
Pollen-----	Over 5	-----	-----	30	3.3	-----	-----	30	.0	-----	-----	30	.0

<sup>1</sup> Isolation interval=7 days<sup>2</sup> Isolation interval=12 days.<sup>3</sup> Isolation interval=13 days.

Two colonies of bees were used. One received contaminated sucrose solution and the other contaminated diluted honey prepared as stated earlier. "Atmospheric" tin-pail feeders with perforated tops were filled with the solutions and inverted, being placed in empty hive bodies over the hives. Except at daily or 2-day intervals when the food supply was replenished, the bees were not disturbed. The feeding was begun on October 6 and the last food was consumed on October 19. An extensive isolation experiment was arranged in which samples of the honey in the comb elaborated respectively from the honey and sugar solutions were incubated in previously sterilized specimen dishes over a range of temperatures varying at 3° intervals from 3° to 24° C.

Since earlier investigators in this field had found that the fire blight organism survives for considerable periods within the hive, the first isolations were not attempted until 7 days after the close of the feeding period. At the first isolation interval the materials were taken from the hives and placed in the dishes in the incubators. From each hive they consisted of 10 transfers of honey and 5 transfers from comb cappings and side walls of cells and scrapings from the frames. At the same time five bees were collected from each hive. From each bee the mouth parts, honey stomach, and alimentary tract were cultured separately. None of the sources tested yielded the bacteria, but the pathogen was readily obtained in all but the last case from samples of the original sugar and honey solutions that had been incubated at 6°, 12°, 18°, and 24° C. Moreover, during the course of the feeding period, the bacteria were successfully recovered at daily intervals up through the twelfth day from the original solutions stored in a shed at approximately 18°, indicating that the pathogenic bacteria survived in the food during that period.

At four subsequent weekly intervals similar isolation series were repeated, always with negative results. At the termination of this experiment, pathogenic bacteria were still being recovered from the original solutions held in the 6° and 12° C. incubators. In these studies the wax, wood, and bee samples were taken from the respective hives at the times indicated. The "honey" at the later weekly intervals was taken from the samples placed in the 24° incubator. These results are interpreted as meaning that the bacteria cannot survive the operations performed by the bees, but precise information was not obtained on the rate of disappearance of the bacteria in the operation of concentrating the food supply. The bacteria apparently had died at a more rapid rate than was expected, necessitating much shorter isolation intervals for determination of the rate of destruction.

#### EXPERIMENT 1933A

In the fall of 1933 the fire blight bacteria were recovered from honey produced from contaminated sucrose and honey solutions within 2 days from the time they had been taken by the bees.

Feeding was begun on October 5, and by October 16 and 18 the respective dilute honey and sucrose solutions were all removed from the feeders by the bees. At the end of the first day one frame was removed from each of the hives and placed in a 30° C. incubator in the laboratory. Near the middle of the feeding period another frame from each of the respective hives was removed and placed in the incubator. In this experiment 40 attempts to isolate the organism were made from the honey, and 20 from each of the wax and frame surfaces at intervals of 1, 2, 3, 4, 5, 6, 8, 10, 12, 14, 18, and 22 days after the bees had taken the contaminated food. Six of the honey isolations made on the first day and six of those made on the second day, or 15 percent of each, yielded the fire blight bacteria. All the others were negative.

Nonpathogenic bacteria were obtained in a large percentage of the isolation tubes of the first six isolation intervals. Their growth in nutrient-dextrose broth was unlike that of the fire blight organism, being much slower and rarely if ever resulting in the formation of a pellicle which is so characteristic of *Erwinia amylovora*. After the first week, when the isolation intervals were lengthened to 2 days, many of the tubes remained sterile and the majority of the others were free of microflora except for occasional molds. All isolations attempted from comb surface for the presence of the fire blight bacteria were negative. Likewise all attempts to recover these bacteria from scrapings of the wood frame were negative with one exception, namely, an isolation attempted on the twelfth day. During the course of this experiment isolations were attempted at the same time intervals from the bees in the two hives. Since the bees were manipulating contaminated food for approximately 2 weeks, a relatively large percentage of the isolations were from bees taken while actually feeding. During this time isolations were tried from the mouth parts, honey stomachs, and alimentary tracts of 100 bees, only 6, or 6 percent, of which yielded the fire blight bacteria. In three instances the bacteria were present both on the mouth parts and in the honey stomachs; in two instances in the alimentary tracts and honey stomachs; and in one instance on the mouth parts alone. Isolations attempted from 10

bees at intervals of 2, 3, 4, 5, and 6 days after the close of the feeding period were all negative. From these data it is apparent that the fire blight organism was not as abundant on the bees as might have been expected. It was further observed that of the positive isolations obtained, there was approximately equal division between the diluted honey and the sucrose solution sources which are considered under one heading in the table.

#### EXPERIMENT 1934A

In the third and final experiment conducted in the fall of the year the fire blight bacteria had disappeared from the honey elaborated from heavily contaminated sucrose solution within 3 days from the time of elaboration by the bees. This experiment, except for a few changes, was similar to experiment 1933a, and the results were in conformity with the previous findings (table 1). The feeding of diluted honey solutions contaminated with the bacteria was omitted because of the uniformity of results of the two solutions in previous trials.

In order to obtain a check on the microflora contributed by the bees and the hive during the concentration of sugar solution the bees were fed sterile sucrose solution for 2 days before they were given the contaminated food. Feeding contaminated food was begun on October 18 and completed on October 25. The number of isolations intervals was reduced, and consisted of 1, 2, 3, 4, 5, and 7 days. The number of isolation trials from the various sources was not changed from the previous corresponding experiment, 1933a. Four series of isolation trials were conducted. The first was confined to the concentrated material from sterile sucrose solution which yielded only molds and miscellaneous bacteria. The other three series were begun on, respectively, October 18, October 23, and October 24. In the first two instances, the frames used in making the isolations were taken from hives and stored in a 30° C. incubator in the laboratory. The third frame was left in the hive and removed only long enough for samples to be taken when it was again replaced. Isolations from the bees were confined to the use of mouth parts and honey stomachs.

Of the 30 isolations made on the first day from the concentrated sucrose solution which had contained fire blight bacteria, 10, or 33.3 percent, were positive for the fire blight organism. From similar numbers of isolation trials on the 2 successive days, there were for each interval two positive, or 6.7 percent. All the later attempts to recover the fire blight bacteria from the concentrated sucrose solution were unsuccessful.

The bacteria were recovered in two cases from bits of wax taken from the combs at the first interval of 1 day, or in 13.3 percent of the attempts. All 70 subsequent trials for the isolation of the bacteria from this source in this experiment were negative. No fire blight bacteria were recovered from 85 samplings of scrapings removed by a sterile scalpel from various parts of the frame.

The bacteria were likewise recovered from 10 of a total of 85 bees, or in 11.8 percent of all attempted isolations made during the time that they were working with the contaminated food. All attempts to recover the bacteria from bees separated from the contaminated feed by more than 1 day were negative. The location of the bacteria in

these bees was chiefly in the honey stomachs, since recovery was made from this source eight times to the three that they were obtained from the mouth parts. From these experiments it is evident that the fire blight bacteria are able to survive in the beehive for a relatively short time.

#### SPRING TRIALS

##### EXPERIMENT 1933S

This study was conducted in the spring of 1933 in the orchard of Cornell University with special cages, which are described later. The honeybees were confined with apple blossoms of the Rhode Island Greening variety. A nucleus beehive was outfitted with two feeders, one containing contaminated sucrose solution and the other contaminated honey solution, one frame of brood ready to emerge, one empty comb, and two cupfuls of worker bees. No queen was included. The nucleus hive was assembled the afternoon of May 13 and installed in the cage in the evening. The hive entrance was opened after dark. During the forenoon of the same day, with the flowers in the late pink stage of development, a lime-sulphur spray (1-40) was applied and in the afternoon an application of nicotine sulphate (1-800), in order to remove insects that might cause complications. Daily observations were made and a record was kept of the behavior of the bees.

A few flowers had begun to open on May 14. At 9 a. m. May 15, the flowers were in early bloom and counts were made of the number of bee visits to groups of 10 blossom clusters in four different cages. For 5-minute intervals there were, respectively, 5, 1, 3, and 4 visits made by the bees, and only an occasional bee was observed trying to gain freedom by flying against the sides of the cage. The flowers came into full bloom on May 17 and the bees were observed freely visiting them up through May 20 at petal fall. The food was all converted into "honey" approximately 5 days before the cages were removed, and the hives were examined on May 26. Immediately after the cages were removed attempts were made to recover the bacteria from the hives and the bees, and also from apple pollen. The first isolations were made 13 days after the beginning of the experiment and approximately 5 days after the feeders were emptied. The results of these isolation trials were all negative but for one instance (table 1) in which the bacteria were recovered from pollen taken from a cell of pollen. Two subsequent series of isolation were made at intervals of 20 and 27 days from the time of starting the experiment, but the results were all negative. The controls, consisting of the contaminated solutions held at 6°, 12°, 18°, and 24° C., all yielded the bacteria at the end of 13 days except the last.

These data support the findings of the autumn experiments, and indicate that the fire-blight bacteria disappear within 5 days from the time contaminated food is converted into honey.

##### EXPERIMENT 1934S

In experiment 1934s, which was a repetition of 1933s, negative results were obtained in all cases for the recovery of the blight bacteria from the nucleus hive. The Twenty Ounce apple variety was here substituted for Rhode Island Greening.



Using the same general procedure as before, the hives were assembled and installed in the cages in the orchard on May 18 and were removed on May 29. On the latter date, isolations were attempted from the same sources as before at approximately 4 days after the last of the food was taken. The fire blight bacteria were not recovered. Two similar isolation series were made at successive 2-day intervals after the first attempts, but no fire blight bacteria were obtained.

#### EXPERIMENT 1935s

The final experiment, which was conducted in the spring of 1935, was a repetition of experiment 1934s.

Bees were placed in the hives on May 21 and the cages were installed on May 22. On June 3, 12 days later, the cages were removed and isolations were tried from the usual sources (table 1). The food had all been consumed approximately 4 days previously. The first isolations attempted were all negative for the fire blight bacteria. Subsequent attempts gave similar results. Isolations from a sample of the infested original contaminated sucrose solution stored at shed temperature (18° C.) yielded pathogenic bacteria to the close of the experiment. The rate of disappearance of the bacteria was not accurately determined in the experiments conducted in the spring of the year because a disturbance to the bees would have altered the experiment in other respects.

#### DISCUSSION

The consistency of the results in all these experiments conducted in the spring and in the fall, in which the bacteria disappeared from the beehive, has an important bearing on the question of the possible role of the bees and the beehive in the dissemination of the fire blight organism.

The rapid disappearance, usually within 2 days, of fire blight bacteria after their introduction into the hive attests to the destructive properties of honey and of the beehive environment, so far as fire blight bacteria are concerned. The two minor exceptions reported above would not appear to invalidate this conclusion, and even for the longest periods of survival encountered, it will be recalled that they were from pollen and scrapings of the wood, in both of which instances there was little opportunity for any possible disinfecting effects of honey to operate. There is, of course, some honey used in the storage of pollen in cells of the comb. The sterilizing action of honey applies as well to other nonspore-forming bacteria previously studied (13) and to the nonspore-forming bacterial flora normally associated with honeybees and the interior of the hive, which survived in honey only a few days longer than the fire blight organism. The relatively short length of life of fire blight bacteria in all the locations studied within the hive probably cannot be ascribed to the sterilizing effects of honey alone or to any other one factor, but is more probably the result of the interaction of a number of factors, including the materials added to sugar solutions by the worker bees in their elaboration of their food supplies. These factors of hive environment consist in part of a high relative humidity (70 percent or even higher during the incoming of fresh food supplies) and of high temperatures (roughly 94° F., within the brood nest of the hive in which most of the elaborated food is stored at least temporarily). Parker (9) has

demonstrated the detrimental influence of high relative humidities on the survival of fire blight bacteria, and, as will be discussed later, high temperatures, within the range of temperatures found within the hive, are also detrimental to their survival. Whether the two factors of destructive relative humidities and high temperatures fully explain the rapid death of the fire blight bacteria within the hive but outside of honey is somewhat questionable, but that they contribute to this end appears evident.

The bees used in these experiments together with the honey they produced were used in cage experiments in the orchard to check further the possibility of the bacteria overwintering in association with the bees that had elaborated the contaminated food.

#### EFFECT OF SUGAR SOLUTIONS ON THE FIRE BLIGHT ORGANISM

A number of workers have studied the effect of sugar concentration on the fire blight bacteria. Experiments by Gossard and Walton (4) demonstrated that the fire blight organism was able to live for about 4 days in honey, for 10 days in aphid honeydew, and for 5 days in peach, plum, and cherry nectar. McLarty (8) stated that fire blight bacteria lived for 48 days in strained honey. According to Parker (9), McLarty stated in correspondence that he later found the bacteria to live for 84 days in the same honey. Thomas (15) reported that the organism causing fire blight lived for 15 days in honey. Using a special nutrient solution as a base, Thomas and Ark (16) determined the maximum concentration of sugars that the organism will tolerate in culture solutions. In dextrose solutions very slight growth was obtained at a concentration of 28 percent while optimum growth occurred at 3 percent. In solutions of levulose and sucrose optimum growth was near 10 percent, but the maximum growth concentrations were, respectively, 18 and 58 percent. The fire blight organism was found to survive for several weeks at 60-percent sucrose concentration. They further point out that the concentrations of the sugars which inhibited the organism corresponded approximately with the osmotic values of the sugars. The longevity of the bacteria in both artificial and natural honey was found by Pierstorff and Lamb (10) to be 5 days.

There is considerable divergence in the results of these various authors. To some extent this may be explained by differences in the degree of individual dispersion of the bacteria within the solutions, for it is evident that if the bacteria were clumped in some cases increased survival might result. It is further to be noted that none of these authors has stated the temperatures at which he worked, and temperature is an important factor in survival of the fire blight organism in sugar solutions.

Because of this lack of agreement in the work of other investigators, tests were made of the survival of fire blight organisms in various sugar solutions, the sugars used being those which occur in nectar and honey, namely, levulose, dextrose, and sucrose, used singly or in combinations.

#### LONGEVITY OF BACTERIA IN SUGAR SOLUTIONS

The longevity of the fire blight bacteria was first tested in the fall of 1932 in artificial nectar (21.2-percent sugar) compounded according to the method of Beutler (1) from sucrose, levulose, and dextrose, of,

respectively, 8.4, 7.3, and 5.5 parts by weight. The sugar solutions were sterilized by filtration through a Berkefeld filter. For supplying the mineral elements a given volume of honey was ashed and a quantity of it corresponding to the amount in nectar was added aseptically to the synthetic nectar solution. A loopful (6 mm) of a dense water suspension of the fire blight organism was added to the artificial nectar. Samples were incubated in flasks at 3°, 12°, and 21° C. Isolations were made at daily intervals for 1 week and at weekly intervals thereafter for 6 weeks. The bacteria were still alive at all the temperatures except 21° at the end of 6 weeks, when the experiment was terminated.

The longevity of the bacteria was tested simultaneously in pure honey, diluted honey, and sucrose solution held at 6° C. The diluted honey and the sucrose solution were samples of the materials fed the colonies of bees in the 1932a experiment. The bacteria survived in these solutions for 31, 20, and 37 weeks. Apparently the low temperature favored their survival.

At the same time, a third experiment was run to test the longevity of the bacteria in duplicate tubes in different concentrations of sucrose solution held at 3° and 21° C. The concentrations tested were 0, 1, 5, 10, 20, 40, and 60 percent of sucrose in water. At 3° the bacteria survived for 28, 9, 8, 8, 6, 10, and 12 weeks, while at 21° the survival periods were 28, 7, 4, 4, 5, 5, and 1 weeks.

A more extensive experiment was begun in December 1932. The sugars tested included sucrose, dextrose, sucrose+dextrose in equal amounts, levulose, synthetic nectar (according to the findings of Beutler (1)), and unsterilized honey. The concentrations used with each material were, respectively, 0, 1, 5, 10, 20, 30, 40, 50, 60, and 75 percent of sugar, each test being made in quadruplicate. The fire blight bacteria were placed in test tubes containing 10 cc of these sugar concentrations held at 6°, 12°, 18°, and 24° C. All except those containing levulose were sterilized in the autoclave for 15 minutes at 15 pounds pressure. To avoid caramelization where levulose was used alone or as a constituent the individual sugars were added to hot sterile distilled water, and heated in the Arnold steamer for 12 minutes on 3 successive days. The inoculum consisted of a highly virulent culture of the organism that had been isolated from crab apple in California. The results of this experiment are contained in figure 1, A. Since no special provision was made for retarding evaporation, there was a marked tendency at 18° and 24° for the sugar solutions to become more concentrated. Thus, for example, the 20-percent sucrose solution incubated at 18° in a period of 35 weeks became concentrated to 59.69 percent sugar. To determine this, examination was made with an Abbe refractometer (drop). Similarly, the 10-percent sucrose solution in 36 weeks became concentrated to 51.22 percent. Obviously this fact should be kept in mind when evaluating these data. In sucrose solution the bacteria survived for a relatively short time in concentrations of 40 percent and above, but there was a discrepancy in the effect of temperature on survival in which 18° appeared to be more favorable than 6°, 12°, and 24°. For dextrose the critical concentrations were 10 percent and above. Mixtures of equal parts of sucrose and dextrose acted much the same as dextrose alone. Levulose appeared to be somewhat less destruc-

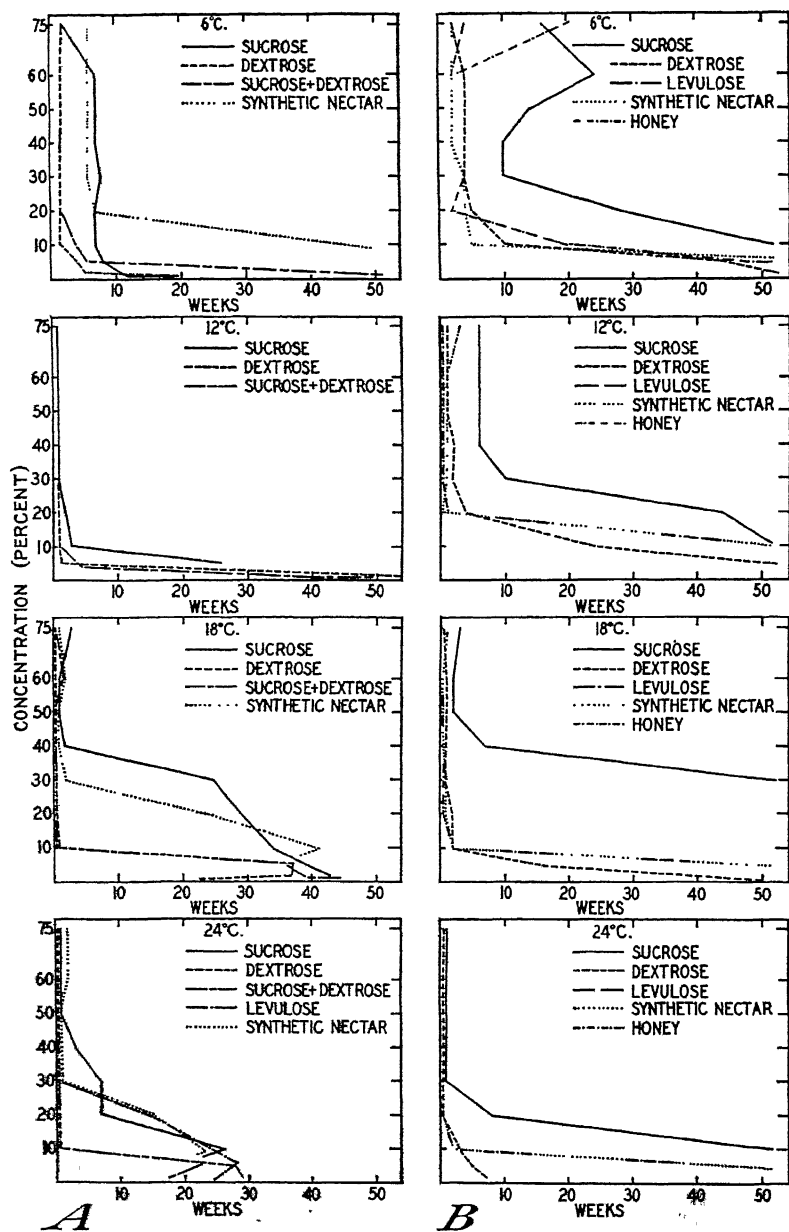


FIGURE 1.—The longevity of the fire blight organism, *Erwinia amylovora*, in solutions of various sugars in different concentrations, and in synthetic nectar and honey held at constant temperatures. The incubation was varied for the two series. *A* involved a single culture and *B* a composite of 30 cultures. The incubation temperatures were 6°, 12°, 18°, and 24° C.

tive of the bacteria than dextrose, but rather similar to sucrose. Synthetic nectar with and without ash behaved more like levulose than like the other sugars. The addition of ash to the synthetic nectar solution appeared to upset the effect of the sugars alone by decreasing markedly the longevity of the bacteria, especially at the lower concentrations, and therefore was omitted in the next experiment. The higher concentrations of all the solutions behaved similarly in killing the bacteria in relatively short periods of time. The survival of the bacteria in a series of unsterilized honey dilutions comparable to the synthetic nectar was similar to the results in the latter. This unsterilized honey series was abandoned at 6 weeks because of contamination by molds. Survival was usually longest at the lower temperatures, but this was not always the case.

Another experiment to test the longevity of the fire blight organism in sugar solutions was begun in November 1933. Certain modifications were adopted, however. A composite culture of 30 organisms, isolated from widely separated places, was used instead of a single culture. The bacteria were added at the rate of approximately 250 million per cubic centimeter determined by use of Petroff-Hausser direct bacteria counter. The respective sugars, sucrose, levulose, and dextrose, were used alone and in combination simulating nectar (1). Extracted honey of approximately 80-percent sugar concentration and the same honey diluted to 60-percent concentration of sugar were also included. No ash was added to the nectar. To reduce evaporation, lead foil was wrapped over the tops of all the tubes held at 18° and 24° C. Before the experiment was begun, the levulose and nectar solutions were heated for 12 minutes in the Arnold steamer on 4 successive days. The sucrose and dextrose solutions were sterilized in the autoclave for 15 minutes at 15 pounds pressure. This experiment was terminated at the end of 1 year; the data obtained are summarized in figure 1, B. From figure 1, B, it is evident that the results correspond to those already discussed in previous experiments. Sugar solutions at concentrations simulating honey (75 percent) and held at temperatures nearest to those which would obtain in the apiary in the summer (24°) are destructive to the bacteria, ordinarily killing them within 1 week. At temperatures more nearly approximating those of the hives in winter (18°), bacterial survival is not sufficient to account for the hive being a potential overwintering source. No definite explanation can be given for the relatively long survival of the bacteria of the composite culture of 30 strains. However, it may be explained in part by reduced evaporation and consequently a lower concentration of sugar.

#### GROWTH OF BACTERIA IN SUGAR SOLUTIONS

Studies were next made of the growth of the fire blight organism in a special synthetic medium (14) when the carbohydrate sources were the respective sugars. The sugar solutions were sterilized by filtration through a Berkefeld filter. In series 1, duplicate tubes were prepared of each of the sugars (sucrose, levulose, and dextrose, and the synthetic nectar mixture) of sugar concentrations of 0, 2, 5, 10, 15, 20, 30, 35, 40, 45, and 50 percent. In series 2, the concentrations were somewhat modified, but covered the same range. Series 3 involved the use of a number of sugar concentrations that differed from each other in smaller amounts as the concentrations approximated

the critical growth limits of the bacteria. The progeny of a single-cell strain of the fire blight organism grown for 2 days on nutrient agar furnished the inoculum. All cultures were incubated at 24° C. The results obtained are summarized in table 2. These data indicate that the monosaccharides, dextrose, and levulose permit a slight growth in sugar concentrations as high as 30 percent and that in sucrose, a disaccharide, the bacteria made a very small but perceptible growth at the 58-percent concentration. These results are in approximate agreement with those of Thomas and Ark (16). When the sugars were combined into a mixture simulating nectar (Beutler (1)), the bacteria grew at 35 percent but not at 40-percent sugar concentration. An abundance of growth was noted at the 5- and 10-percent concentrations, as evidenced by the sediment at the bottom of the tubes and the color change of the medium, Bromothymol blue was the indicator used. At the higher concentrations growth decreased with increasing concentration. No growth appeared at the 40-percent concentration.

To account for the rapid disappearance of the fire blight bacteria during the process of elaborating nectar into honey several experiments were tried.

TABLE 2.—Summary of studies conducted for the purpose of finding the maximum concentrations of various sugars, and of synthetic nectar, at which the fire blight organism, *Erwinia amylovora*, will grow

Series no. and sugar	Growth of <i>E. amylovora</i> at the end of 10 days in a synthetic medium containing sugars in the indicated concentrations <sup>1</sup>												
	0 per cent	2 per cent	5 per cent	10 per cent	15 per cent	20 per cent	25 per cent	30 per cent	35 per cent	40 per cent	45 per cent	50 percent	
Series 1:													
Dextrose.....	—	+	+	+	+	+	+	+	—	—	—	—	
Levulose.....	—	+	+	+	+	+	+	+	—	—	—	—	
Sucrose.....	—	+	+	+	+	+	+	+	+	+	+	+	
Nectar.....	—	+	+	+	+	+	+	+	+	—	—	—	
	0 per cent	3 75 per cent	7 5 per cent	15 per cent	18 75 per cent	22 5 per cent	26.25 per cent	30 per cent	35 per cent	40 per cent	45 per cent	50 percent	
Series 2:													
Dextrose.....	—	+	+	+	+	+	+	+	—	—	—	—	
Levulose.....	—	+	+	+	+	+	+	+	—	—	—	—	
Sucrose.....	—	+	+	+	+	+	+	+	+	+	+	+	
Nectar.....	—	+	+	+	+	+	+	+	tr.	—	—	—	
	0 per cent	5 per cent	10 per cent	20 per cent	25 per cent	28 per cent	30 per cent	32 per cent	35 per cent	40 per cent			
Series 3:													
Dextrose.....	—	+	+	+	+	+	+	—	—	—	—	—	
Levulose.....	—	+	+	+	+	+	+	—	—	—	—	—	
Nectar.....	—	+	+	+	+	+	+	+	+	—	—	—	
	0 per cent	5 per cent	10 per cent	20 per cent	30 per cent	40 per cent	48 per cent	50 per cent	52 per cent	54 per cent	56 per cent	58 per cent	60 per cent
Sucrose.....	—	+	+	+	+	+	+	+	+	+	+	+	—

<sup>1</sup> + Growth; — No growth. The synthetic medium was made according to the formula given by the Society of American Bacteriologists (14).

The effect of desiccation alone on the longevity of the bacteria in sugar solution cannot account for their rapid disappearance in the honey-ripening process. A contaminated sucrose solution of 60-per-

cent concentration was placed in Petri dishes in a desiccator ( $\text{CaCl}_2$ ) and kept there until the concentration of sugar was between 79 and 80 percent. The dishes were sealed and placed in a  $30^\circ\text{C}$ . incubator and were removed only for periodic examination. It was found that when 10, 20, 30, or 40 cc of the solution was placed in four Petri dishes of equal size the time required for the desiccator to adjust to the desired concentration was approximately 1, 2, 3, and 4 days. At the end of 1 month, when the isolation trials were discontinued, the bacteria were still being recovered from all the plates except the second. In this plate, the bacteria were still present at the end of 3 weeks. Portions of the original sugar solution were simultaneously incubated at the temperatures  $6^\circ$ ,  $12^\circ$ ,  $18^\circ$ ,  $24^\circ$ , and  $30^\circ$ . At the end of 1 month the bacteria were still alive at  $6^\circ$  and  $12^\circ$ , but had disappeared at  $18^\circ$ ,  $24^\circ$ , and  $30^\circ$  by the end of 18, 12, and 6 days, respectively.

When the experiment was repeated with sucrose solution, synthetic nectar solution, and similarly diluted honey of 21.2-percent sugar concentration as the starting point, comparable results were obtained. The rate of disappearance of the bacteria from these solutions slightly greater than for the sucrose solution, but cannot account for the rapid disappearance within approximately 2 days of the bacteria found in the feeding experiments detailed earlier.

An abundant microflora was found to be associated with the honeybee and the hive interior. Although these bacteria outlive the fire blight organism in honey, they too, disappear at the end of approximately 1 week. Preliminary studies on antagonism between these bacteria and the fire blight organism when grown on agar and in broth gave some indication that the former may function in some way in inhibiting the latter, but the evidence is too limited to justify its being given in detail.

#### THE FIRE BLIGHT ORGANISM AND THE LIFE CYCLE STAGES OF THE INSECT

Attention was next directed to the possibility that the bacteria might be associated with honeybees in some stage of the life cycle other than that of the active adult.

Two series of experiments were conducted in October 1933 and 1934. The same colonies of bees were used as in experiments 1933b and 1934b, already discussed. Shortly after the beginning of the feeding periods the queen bees, in response to the influx of food, began to deposit eggs in the cells, and continued to do so throughout the feeding period and for a short time afterward. As soon as the eggs hatched (3 days), the young larvae obtained their nourishment from a contaminated sugar solution or from the elaborated product, the only food available, and food of a contaminated source continued to be their diet until pupation (to the 9th day). In 21 days, or after 12 days as pupae, the adults emerged.

For making isolation studies from the immature life-cycle stages of the honeybee, small test tubes (1 by 6 cm) were fitted with cork stoppers into which round, stiff, toothpicks had been inserted. This type of pick was rigid and suited for loosening and removing specimens from the cells.

A frame containing brood was removed from the hive and the specimens were taken and stored in the test tubes for transporting to

the laboratory. A sterile scalpel was used for opening capped cells. For making isolations all specimens except the eggs were placed in a Petri dish containing a small amount of sterile water, chopped fine, and allowed to stand for about 10 minutes. A large loopful (6 mm) of the material was then transferred to broth and the usual procedure followed. In the isolations from the eggs a modified procedure was used. An egg was first allowed to stand for 10 minutes in water. After sampling the washings for the presence of the bacteria, the egg was crushed and handled in the same manner as the other stages. Several parallel isolation series were run in which the insect stages were placed directly in the broth.

In a preliminary experiment during the latter part of the feeding period in 1933, two series of isolations were attempted from, respectively, 10 eggs and 10 small larvae. One of the larvae in the first series yielded fire blight bacteria while the others were negative. Though limited in scope, this study indicated that the larval stage was receiving food containing the bacteria, although it is not known just when in the process of elaborating honey it was given to the larvae.

In the 1933 experiment the isolation intervals were 12, 14, and 17 days after the contaminated food had all been consumed, and in the 1934 experiment the isolation intervals were 15, 17, and 19 days. Both in 1933 and 1934, bacteria other than the fire blight organism were frequently obtained from the larval stage, but relatively rarely from the egg, pupal, and unemerged adult stages. The fire blight bacteria were not recovered in any instance in the regular series of tests after an interval of at least 12 days after the contaminated food had been taken, which is strong evidence that these bacteria are not associated with the life-cycle stages of the insect.

#### DISSEMINATION OF FIRE BLIGHT BACTERIA FROM THE BEEHIVE TO FLOWERS

The dissemination of *Erwinia amylovora* from the beehive to flowers caged with honeybees has been studied by several investigators. Gossard (2) postulated that if one bee carries 100,000 bacteria into the hive 1 day, on the following 1 or 2 days each of 1,000 bees has the potentiality of carrying a considerable fraction of 100 virulent bacteria out to fruit blossoms. He postulated further that the bacteria may multiply in the raw nectar when first carried into the hive. Pierstorff and Lamb (10), in studies conducted in Ohio, atomized the interiors of beehives with bouillon cultures of the fire blight organism, and concluded that: "Bees did not carry blight inoculum from the hive to the blossom clusters" when "beehives that had been infested with virulent cultures of *Bacillus amylovorus* were placed under apple trees enclosed within cheesecloth cages." However, they did get a small amount of infection possibly originating from the hive, which they attributed to factors incidental to the use of cheesecloth tents, such as rents or tears and splashing rain from diseased trees nearby. Thomas and Vansell (19) working with caged pears in California, found that when beehives were transferred from the diseased to healthy trees, no infections resulted. The methods of handling the bacteria may in part account for the discrepancies in these results.



Since it was desired to control the experimental conditions as closely as possible, small cages were used that fit over individual branches containing between 100 and 300 blossom clusters (fig. 2). The frame consisted of chicken wire of 1-inch mesh and 4 feet wide. A strip about 6½ feet long was folded to make a cylinder 4 feet long and approximately 2 feet in diameter. Wire screen and two types of transparent waterproof materials, consisting of waterproof cellophane impregnated over cloth and wire screen, were used as covers. For closing the cages over the branches, strips of unbleached muslin of about 40 meshes to the inch and 2 feet wide were sewed to the cages at either end. Ventilation was provided by inserting two pieces of wire screen 6 inches square into the cloth at either end, the inside one on the upper and the outside one on the lower side. Two strips of lath made to fit into the wire mesh of the support were used at either end of the cage for spreading the cage and transferring its weight to the branch. The nucleus hive of bees was suspended from the limb at the end of the cage nearest the tree.

The nucleus hives, unless otherwise specified, were made up to include: (1) Two feeders filled with sucrose solution, either sterile or contaminated with a water suspension of the fire blight bacteria; (2) one frame with brood ready to emerge; (3) one frame of sealed honey for emergency food supply; (4) one frame with empty comb to serve as a depository for pollen and for honey produced from sugar solution and nectar; and (5) 2 cupfuls of bees (approximately 500).

The cages were installed between the pink and early bloom stages of unfolding of fruit buds and were kept on until petal fall was completed and sufficient time had elapsed for disease symptoms to appear. Before installing the cages, as detailed earlier in experiment 1933s, special precautions of spraying were taken to control insects that might interfere with the experiment. The bees and food involved each season were from the same sources, except when specified otherwise, and were as uniform as possible. Bees from the same hives were used for the three seasons. To insure setting of fruit in the final of the 1935 series, a bouquet of McIntosh, a suitable pollinizer, was used to pollinize Rhode Island Greening blossoms in each cage containing bees.

Oozing cankers were prepared by inoculating succulent Bartlett pear shoots in the greenhouse and transferring these shoots when oozing copiously to flasks containing water which were suspended among the blossoms. To keep the interior temperatures of the rain-proof cages near those outside, the cages were placed on the shady side of the trees and shade was provided when necessary.

In addition to investigating the transmission of the fire blight bacteria from the beehive to apple blossoms, studies were made of related aspects. For example: (1) The honey produced from contaminated food in the fall of the year was fed to a nucleus hive of bees housed in one of the enclosures to further check on the possibility of bacteria overwintering in the hive. There were also used bees from the same colony that had produced this honey. (2) As a check on (1), another hive with similar bees was fed sterile sugar solution alone; (3) the relation of bees to the oozing canker or overwintering source of fire blight inoculum was tested by caging bees with an

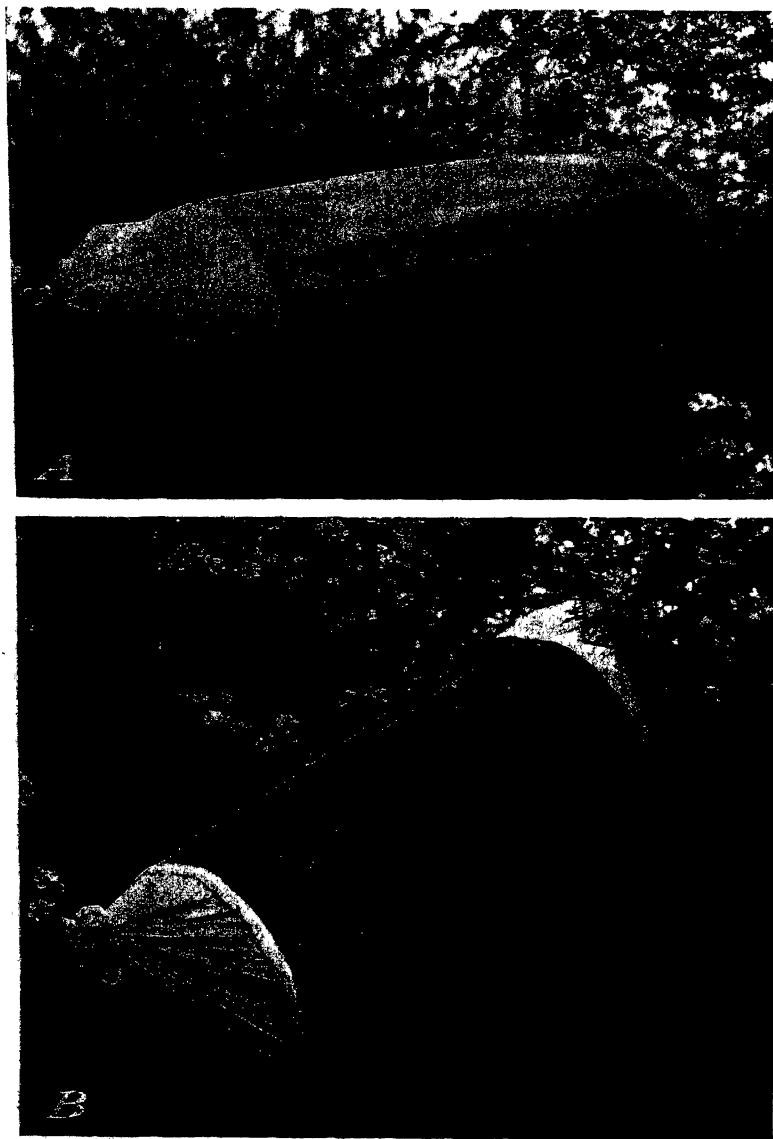


FIGURE 2.—Special cages for the study of the factors involved in the dissemination of the primary and secondary inoculum of fire blight. A, A waterproof cage designed to exclude such factors as insects and rain from the enclosed branch, used in studies of several factors such as insects, insect food, and cankers, alone or in combination; B, a screen-covered cage designed to exclude pollinating insects but to permit the entrance of rain, and adapted for studying the factors of rain, insects, and cankers in various combinations.

oozing canker; (4) a check on (3) was made by placing an oozing canker alone in a cage; (5) rain as a dissemination factor was tested by enclosing an oozing canker in a screen cage which permitted the access of rain; (6) a check for rain alone was employed; and finally (7) bees plus an oozing canker were enclosed in a screen cage admitting rainfall. These studies are summarized in table 3.

TABLE 3.—*Results of cage experiments conducted in the orchard to determine the role of the honeybee in the overwintering, primary spread, and secondary spread of the fire-blight organism*<sup>1</sup>

Factors studied	Year	Blossom clusters				
		Total	Healthy	Blighted		Setting fruit <sup>2</sup>
		Number	Number	Number	Percent	Percent
Bees + old honey made from contaminated food in preceding October.....	1933	214	214	0	0.0	-----
	1934	175	175	0	0	-----
	1935	199	199	0	0	32.0
	1933	219	205	14	6.3	-----
Bees + contaminated sucrose solution.....	1934	202	200	2	1.0	-----
	1935	204	162	52	25.5	30.0
	1933	-----	-----	-----	-----	-----
	1934	210	210	0	0	-----
Bees alone, check.....	1935	204	204	0	0	28.0
	1933	112	105	7	6.3	-----
	1934	168	168	0	0	-----
	1935	176	176	0	0	0
Rain alone, check.....	1933	-----	-----	-----	-----	-----
	1934	244	244	0	0	-----
	1935	162	162	0	0	0
	1933	211	211	0	0	-----
Bees + oozing canker.....	1934	171	171	0	0	-----
	1935	151	151	0	0	18.0
	1933	208	191	17	8.2	-----
	1934	219	219	0	0	-----
Oozing canker + rain.....	1935	131	129	2	1.5	0
	1933	215	4	211	98.1	-----
	1934	202	201	1	5	-----
	1935	158	155	3	2.0	25.0

<sup>1</sup> The cages were installed and removed on the following dates: In 1933, May 13 and 26; in 1934, May 18 and 29; in 1935, May 22 and June 3. All the cages, except where the factor for rain was involved, when a screen cage was employed, were made of a transparent waterproof material. Rainfall during the experimental periods was in 1933 on May 13, 0.06 inch, May 20, 0.42 inch and May 24, 0.52 inch; in 1934 on May 22, 0.01 inch and on May 24, 0.09 inch; and in 1935 on May 28, 0.09 inch and May 29, 0.03 inch.

<sup>2</sup> Pollentizers were introduced into the cages only the last year, but in the preceding 2 years as well as in the last year a record was kept of the visits made by the bees to flowers for time intervals of 5 minutes on succeeding days. These data, although not included here, show that the bees visited flowers in all cages.

Blossom blight developed in cages where the bees were fed freshly contaminated sucrose solution. This is evidence that the bacteria were carried from the beehive to the flowers to bring about the infection, since no other known source of the bacteria was available. However, it is not possible to state how many bees were involved, or whether the sucrose solution had first been concentrated. It is probable that certain of the 1935 infections resulted from secondary spread by the bees after some bacteria had been carried out of the hives to the flowers, for from June 3 to June 9 the number of blighted clusters increased from 43 to 52, or approximately 20 percent.

No blossom infections occurred in the cages containing honeybees and honey for food that had been produced the previous autumn from contaminated sucrose or honey solution. These data confirm the results of the isolation experiments in which it was found that the fire blight bacteria quickly disappear from honey elaborated from heavily contaminated food.

Cage experiments to check the possibility that the bacteria may overwinter in the bees themselves gave negative results. The bees used in these experiments had consumed contaminated food the previous fall and were given sterile sucrose solution while in the cage. These results confirm those of the experiments in which it was found that the bacteria were not associated with the life-cycle stages of the insect.

No blossom infections resulted from caging bees with oozing cankers. This finding is in conformity with the observations of a number of workers, including Jones (6) and Thomas and Parker (18). Apparently honeybees are not attracted by the bacterial ooze of the fire blight disease. In similar cages containing only the oozing cankers, there were likewise no infections.

Rain was found to be a factor in the dissemination of fire blight bacteria from oozing cankers to the neighboring blossoms. In 1933 the rainfall, which approximated 1 inch during the experimental period, was sufficient to cause the infection of 8.2 percent of the caged blossoms. The blighted blossom clusters were all in the immediate vicinity of the oozing canker. Subsequent trials in 1934 and 1935 resulted in practically no infection because of the lack of sufficient rainfall while cankers were in the cages, it being only 0.10 and 0.12 of an inch for these years.

In the check for rain alone for 1933, 6.3 percent of the blossom clusters were blighted. These infections were traced to a canker in the tree above and slightly to one side of the cage. In the 2 succeeding years, no infections were encountered in similar experiments.

When the factors of oozing canker, rain, and bees were combined in the same cage, the percentage of infected blossom clusters for the three seasons were, respectively, 98.1, 0.5, and 2.0. The rainfall was sufficient only in 1933 to disseminate some of the bacteria from the oozing canker to the flowers in the primary spread of the organism. The large percentage of blossom clusters infected demonstrates the role of the honeybee as a secondary spreader of the bacteria from flower to flower. The isolated infections in 1934 and 1935 may be accounted for by the possible direct contact between blossoms and oozing canker because of their close proximity. The results of these experiments on the role of the honeybee as a disseminator of secondary inoculum are in agreement with the findings of Waite (20), Gossard and Walton (4), Thomas and Vansell (19), and Pierstorff and Lamb (10).

From these data, it appears that the honeybee may function in two ways,<sup>7</sup> namely, (1) in carrying the bacteria from the beehive to flowers, and (2) in the secondary spread of fire blight inoculum from flower to flower. The transmission of inoculum in the first instance is conditioned, however, on the bees actively feeding on food contaminated with the fire blight organism. It would seem probable during years of blight epiphytotics that the causal bacteria may be carried out from the hive and initiate infections, with the insect apparently functioning purely in an accidental capacity. These data confirm the results of the isolation experiments discussed earlier in which bees feeding on contaminated food harbored the organism for

<sup>7</sup> The honeybee may also disseminate the bacteria in another way. Unpublished data show that the causal bacteria may infect pollen in blighting flowers even before anther dehiscence. Pollen-gathering bees carry such pollen from flower to flower and infection results when it lands on the stigmas of flowers, even when the relative humidity of the surrounding atmosphere is extremely low.

no longer than 1 day. The percentage of bees functioning in this capacity was relatively small (table 1), which would seem to indicate that the hazard of infections from this source is not great.

#### SUMMARY

The results of studies to determine the role of the honeybee and beehive in the development of the fire blight disease indicate that the fire blight organism is incapable of overwintering in the beehive or in association with the honeybee. Therefore these locations cannot constitute a source of primary inoculum in the spring of the year.

This investigation extended over a period of four seasons and had several objectives, namely, (1) to discover the longevity of the bacteria causing fire-blight in the beehive when introduced through the natural channel in the food of the bees, (2) the longevity of the bacteria in sugar solutions over a range of concentrations and temperatures, (3) the location of the bacteria in relation to the life cycle of the insect, and (4) the extent of dissemination of the bacteria from the beehive to flowers in cages in the orchard.

When introduced through the food of the bees, the fire blight bacteria were not reisolated after 3 days from such locations as honey, comb, frame, and bees. Only in two instances were the bacteria recovered after 3 days from the time the contaminated food was taken, that is, from pollen at 13 days and from frame scrapings at 12 days.

The longevity of the bacteria in the sugar solutions varied with the incubation temperature, the sugar concentration, and the sugars used. These data provide evidence that the survival of the bacteria in the beehive is highly improbable at the temperatures and sugar concentrations normally encountered in the apiary. The bacteria survived for relatively short intervals (from 1 day to several weeks, depending on the kind of sugar and the incubation temperature) at sugar concentrations of 30 percent and above, covering the range of heavy nectar to honey that would be found in the beehive. The bacteria ordinarily survived longest at the lower temperatures. Of the materials used, sucrose solution had the least bacteriostatic and bactericidal effect on the bacteria. When a synthetic culture solution was used as a base, the maximum sugar concentrations at which the fire blight organism grew in dextrose, levulose, artificial nectar, and sucrose were, respectively, 30, 30, 35, and 58 percent.

The fire blight bacteria were not found associated with the immature stages of the life cycle of the honeybee even though an abundance of the bacteria were present in the food of the bees at all times during the rearing of the brood.

The honeybee was found to function in two ways, namely, (1) in carrying the bacteria from the beehive to flowers, and (2) in the dissemination of fire blight inoculum from flower to flower. Transfer of inoculum from the beehive to flowers was conditioned, however, on the bees actively feeding on contaminated food.

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## LENGTH OF THE DORMANT PERIOD IN CEREAL SEEDS <sup>1</sup>

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### INTRODUCTION

Cereals from a great variety of sources have been used in hybridization experiments to improve the yield and quality of the grain. Little or no account appears to have been taken, however, of the factor of dormancy in the varieties used for crossing. Doubtless parent plants grown under different climatic conditions often differ in many other factors than those desired to combine in breeding, and these characters are not easily determined by the yield and quality of the grain or the anatomical structure of the plant. This paper deals with one such character—the heritable differences in the length of the rest period.

In this study all types of summer and winter cereals, grown as nearly as possible under the same conditions, were used. Because of the large number of varieties employed it was necessary to use seed grown in field plots. The use of field-grown material has some practical advantages, for the effects on dormancy produced by conditions in the field may differ from those produced under controlled conditions in the greenhouse.

### METHODS

Samples for testing were collected from cereals growing on field plots at University Farm, St. Paul, Minn. The plants were grown under fairly uniform conditions.

Since it was known that the stage of maturity of the seed affects the length of its rest period, samples were collected at three stages of maturity—soft dough, hard dough, and ripe. It was not possible to determine these stages accurately. Collections of all varieties of a certain stage of maturity generally were made on the same date, which is recorded together with the dates of the germination trials. Spring wheats were collected later than winter wheats. Collections were made of early and of late oat varieties.

It was thought that temperature might affect the length of the rest period, and each collection was accordingly stored at electrically controlled constant temperatures, as follows: 0°, 10°, and 20° C. during the experiment of 1929; 0°, 7°, and 14° in 1930; and 0°, 10°, 20°, 30°, and 35° for a few varieties in 1931. All the samples were stored in paper bags and kept in darkness except for such periods as

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they were required for sampling. The humidity of the storage rooms was not determined or controlled. The storage temperature was electrically controlled to within  $\pm 1^\circ$ . When doors were opened, the temperature sometimes rose as much as  $2^\circ$  to  $3^\circ$  for a few minutes.

The probable response of the cereals to conditions of high humidity in the shock or stack for short periods after rains is indicated by the rapidity of sprouting in the germination tests. However, these tests were all made at one temperature,  $20^\circ\text{C}$ ., and it was not therefore possible from these data to combine the effects of high humidity with different temperatures in germination. Higher or lower temperatures combined with high humidity in wet shocks or stacks may be expected to modify the germination rate.

Germination tests were made in electrically controlled germinators which varied less than  $1^\circ$  from  $20^\circ\text{C}$ . The tests were made under the standard conditions recommended by the Association of Official Seed Analysts of North America,<sup>3</sup> and the equipment of the Minnesota State Seed Laboratory was used. Germination counts were made on the third, fifth, and seventh day of the test to indicate the speed of germination. Tests were made at intervals to determine the length of the dormant period for each variety. The dates of collection and determination of percentage germination are recorded, since the date of ripening differs from year to year.

#### PRESENTATION OF DATA

That there are great varietal differences in the length of the rest period in cereals not determined entirely by ecological or physiological conditions is shown by the data in tables 1 to 14. The lower the temperature of storage, the longer is the rest period. Cutting the plants while still unripe generally increases the length of the after-ripening period.

Among the barleys (tables 1-3) there was a considerable difference in the rate of germination. The variety with the longest rest period was Trebi 448. When the plants were cut ripe only 8 percent of the seed germinated (table 2) after 73 days' storage at  $0^\circ\text{C}$ ., 18 percent after 73 days' storage at  $7^\circ$ , 10 percent after 27 days at  $14^\circ$ , and 100 percent after 52 days at  $14^\circ$ . Colcess 461 also had a rather long dormant period. Manchuria, Velvet, and Peatland had very short rest periods when stored at  $14^\circ$  even if cut in the soft-dough stage.

Nearly all of the rye varieties (tables 4-6) showed a high germination percentage when cut ripe and stored at  $14^\circ$ . Green Select 102 and Prolific had the longest afterripening periods. Montagney seems to be characterized by a much shorter afterripening period than other varieties. This is evident in early cuttings. When plants of this variety were cut in the soft-dough stage and the seed was stored for 30 days 78 percent of that stored at  $0^\circ$  germinated and 88 percent of that stored at  $14^\circ$ .

Oats (tables 7-10) cut in the ripe stage generally had only a short rest period. Gopher oats cut ripe finished their afterripening and germinated completely within 26 days. If a shorter time of storage should be taken, it would be difficult to separate the effects of the

<sup>3</sup> UNITED STATES DEPARTMENT OF AGRICULTURE. RULES FOR SEED TESTING. Dept. Cir. 406, 13, pp. 1927.



storage temperature from the effects of the temperature during germination. When oats are cut green, the rest period is increased, and the seed shows less tendency to sprout immediately. Low temperatures further increase the length of the rest period.

Winter wheats (tables 11-13) showed great varietal differences in the length of their rest periods. Allowing the wheat to become fully ripe decreased the time required for afterripening. Winter wheats generally had a shorter rest period than spring wheats. Two separate crosses, Minard  $\times$  Minhardi no. 2313 and no. 2314, to form the same hybrid between two winter wheat varieties, showed great similarity in their rest periods. Minturki wheat has a shorter rest period than Minard, which has a shorter rest period than Minhardi. The cross Minhardi  $\times$  Minturki has a shorter rest period than Minard  $\times$  Minhardi.

Minturki seed at 14° germinated with great energy after 23 days' storage, whether cut in the soft-dough, hard-dough, or ripe stages, but lowering the storage temperature to 0° decreased the rapidity of germination. Lowering the storage temperature to 7° decreased the rapidity of germination of that cut in the ripe stage.

When Marquis wheat has completed its rest period, the rapidity or energy of germination increases; that is, a high percentage germinates after the seed has been held under conditions favorable for germination for 3 days.

Among the spring wheat varieties (tables 11, 12, and 14), some have a notably long rest period, failing to germinate until so late in autumn that if planted out of doors in Minnesota temperature conditions would prevent their germination and enforce a dormancy until spring. Mindum 470, Marquillo 2202, Kubanka 2310, and Double Cross 2303, 2304, and 2305 have notably long rest periods. After storage for 2 months at freezing temperatures, they give practically no germination. At 7° storage the rest period is more than 2 months, and at 14° it is about a month. Double Cross 2315 and 2316 showed a slightly higher germination percentage on the third test than Double Cross 2303.

When cut ripe and stored at 14°, the rest period is about 23 days for Kubanka 2310, Reward 2204, Supreme 2309, Ceres 2223, and Reliance 2308.

From the results of 1929 and 1930 it seemed desirable to test at shorter intervals the germination of some varieties that had shown a very short rest period and to extend the range of storage temperatures to 30° and 35°. This was done in 1931 with a few varieties of each cereal. The results of the first 2 years indicated that at low temperatures the importance of the length of the rest period was emphasized. In the experiments of 1929 and 1930 the temperatures used were such as occur after harvest time in northern Minnesota in certain years. In other years, however, the period following harvest, is very hot, and it was thought that temperatures of 30° and 35° probably would not be above those that might obtain at times in cereals in the shock.

In winter wheat (table 13) collected in the hard-dough stage, the germination percentage was higher after 2 days storage at 0°, 10°, and 20° C. than after 9 days (second test) or 20 days (third test) storage at these temperatures. Evidently the seed had not gone into its deepest rest when collected in the hard-dough stage, and required about

9 days to reach maximum dormancy. When stored at 30° or 35°, this was not the case, and the percentage germination increased with time of storage except in the case of Minhardi, which did not consistently increase. When the seed was collected ripe, this decrease in germination was not found. Storage of Minturki wheat, in the hard-dough stage, at 30° for 9 days was as effective in breaking the rest period as storage for more than 40 days at 0° or 10°. At 20° the time required to reach 90 percent germination (7-day count) was 30 days. For Minhardi × Minard and Minhardi × Minturki the reactions to storage temperatures were somewhat similar to those of Minturki. Storage for only 2 days at all of the various temperatures gave nearly the same percentage germination of winter wheat collected in the hard-dough stage as can be expected for such a short period of storage; in fact, the germination period at 20° was longer than the storage period. Tests at such a short storage interval as 2 days were made only on the hard-dough stages of winter wheat and rye. The other samples were put directly under germinating conditions at 20° on the day of collection. The values of this test were taken as the original germination percentage and tests were not again made until the lapse of about 1 week.

In the data on spring wheat (table 14) collected in the hard-dough stage, there is also some indication of a decrease in germination percentage on the second test. This increase in depth of the resting condition is indicated also in collections in the hard-dough stage in rye (table 6), oats (tables 9 and 10), and barley (table 3), but is generally not shown in the collections of ripened grain.

The effect of the higher temperatures in speeding up the after-ripening processes is noticeable especially in collections in the hard-dough and ripe stages of winter and of spring wheats, oats, and barley. In certain cases the higher storage temperature seem to have decreased germination on the second test of rye collected in the hard-dough stage. The greater length of the rest period of Prolific 89 rye is marked.

In oats a rest period of any considerable length was found only in the late varieties. The rest period was longer in North Dakota 20014 than in any other variety. Cutting early or in the hard-dough stage is indicated as a possible means of increasing the length of the rest period, but this might be effective only if the temperature is low, for the rest period is quickly passed at high temperatures.

#### SUMMARY

The length of the rest period of common varieties of wheat, oats, barley, and rye was determined by germination tests at three stages of ripeness: Soft dough, hard dough, and ripe. The rest period was found to be longest in immature seeds. Lowering the temperature at which the seed was stored generally increased the length of the rest period. The length of the rest period varied greatly with the variety. Generally speaking, winter wheats had a shorter rest period than spring wheats.

TABLE 1.—Percentages of germination of barley varieties harvested at different stages of maturity and stored at different temperatures, 1929

[100 seeds taken for each test]

Storage temperature and variety	Germination at soft-dough stage, collected July 17, after storage for indicated number of days						Germination at ripe stage, collected July 26, after storage for indicated number of days					
	First test <sup>1</sup>		Second test <sup>2</sup>		Third test <sup>3</sup>		First test <sup>1</sup>		Second test <sup>2</sup>		Third test <sup>3</sup>	
	5	7	5	7	5	7	5	7	5	7	5	7
0° C.:												
Manchuria × South African 457..	1	15	0	8	32	40	0	2	2	10	19	21
Mechanical mixture.....	1	8	2	3	22	24	7	10	2	8	77	81
Manchuria 184.....	10	20	18	24	28	28	0	1	3	6	85	85
Composite crosses 41.....	2	5	5	25	24	32	4	8	0	2	71	77
Colseas 461.....	0	2	0	3	12	17	0	1	4	6	7	10
Svanhals × Lyon 474.....							12	33	5	20	53	67
Small awn × Manchuria 412.....							4	5	5	13	27	34
Heinrich's 465.....	3	36	28	63	91	97	0	1	62	65		
Glabron 445.....	0	2	0	3	3	6	1	3	2	8	18	28
Svansota 440.....	3	10	0	5	27	33	4	4	1	7		
Velvet 447.....	2	16	4	18	96	97	5	12	8	12	88	88
Trebi 448.....	3	19	0	12	45	56	1	2	6	23	54	66
Peatland 452.....	4	31	9	27	80	97	7	9	83	88	100	100
Minsturdi 439.....	4	16	28	51	87	89	3	5	16	22	94	95
10° C:												
Manchuria × South African 457..	1	2	3	7			1	8	7	15		
Mechanical mixture.....	0	0	1	2			2	6	25	31	100	100
Manchuria 184.....	3	5	13	14			12	14	21	24	98	98
Composite crosses 41.....	1	3	10	14			2	12	24	38		
Colseas 461.....	1	1	2	3			1	3	27	30	6	22
Svanhals × Lyon 474.....							1	7	3	11	89	89
Small awn × Manchuria 412.....							2	3	8	15		
Heinrich's 465.....	0	0	26	29			0	0	19	31	93	93
Glabron 445.....	0	0	1	2	37	41	0	1	8	10	70	72
Svansota 440.....	0	0	10	11	24	25	0	1	35	49	99	99
Velvet 447.....	0	0	11	13			4	10	49	64	98	98
Trebi 448.....	0	1	3	13	30	41	1	2	2	8	33	64
Peatland 452.....	0	2	29	33			4	5	93	95		
Minsturdi 439.....	1	8	28	46			0	13	61	69		
20° C:												
Manchuria × South African 457..	9	16	91	94			3	13	83	84		
Mechanical mixture.....	1	1	59	63	100	100	1	2	86	87		
Manchuria 184.....	4	6	75	75			4	6	75	75		
Composite crosses 41.....	15	26	38	41	94	94	7	13	66	73		
Colseas 461.....	6	10	31	43	58	62	3	3	1	8	58	70
Svanhals × Lyon 474.....							4	9	78	82		
Small awn × Manchuria 412.....							4	6	79	83		
Heinrich's 465.....	6	7	100	100			2	6	98	99		
Glabron 445.....	4	6	58	62			1	2	59	67	95	95
Svansota 440.....	2	3	100				0	4	99	100		
Velvet 447.....	2	2	100	100			2	11	98	98		
Trebi 448.....	9	14	27	35	58	73	1	3	98	99		
Peatland 452.....	43	46	100	100			70	74	100	100		
Minsturdi 439.....	4	23	16	37			2	17	100	100		

<sup>1</sup> Begun Aug. 8, ended Aug. 15.<sup>2</sup> Begun Sept. 5, ended Sept. 12.<sup>3</sup> Begun Sept. 25, and Oct. 2.



<sup>14</sup> O C.																										
Manchuria × South African 457.....	0	2	12	42	62	70	100	---	---	0	14	22	98	98	98	98	90	90	90	86	96	98	96	100	---	
Mechanica mixture.....	0	0	2	24	28	36	96	96	96	98	2	4	12	100	---	98	100	---	92	100	---	100	---	---	---	
Manchuria 184.....	0	4	10	78	80	84	92	96	100	12	44	52	98	98	98	98	100	---	98	100	---	100	---	---	---	
Composite crosses.....	0	0	4	38	44	48	92	96	100	---	8	16	98	98	98	96	98	---	98	---	---	---	---	---	---	
Cosses 461.....	0	0	0	0	6	6	94	96	96	0	10	12	56	70	74	70	84	---	8	26	100	---	94	94		
Svanthalis × Lion 474.....	0	4	4	6	14	24	100	---	---	0	8	10	100	---	100	---	---	---	---	---	---	---	---	---	---	
Svanthalis × Lion 475.....	0	0	6	12	22	100	---	---	---	0	6	12	98	98	100	94	96	---	98	100	---	100	---	---	---	
Small ewn × Manchuria 412.....	0	0	0	10	12	14	100	---	---	0	0	0	98	98	100	100	---	---	---	---	---	---	---	---	---	
Hedrichs 466.....	0	0	0	62	78	94	98	98	98	2	24	36	100	---	100	---	---	---	---	---	---	---	---	---	---	
Glabron 445.....	0	2	8	14	28	50	98	98	98	0	6	6	96	96	96	92	96	---	96	100	94	98	98	100	---	
Svansota 440.....	0	4	8	58	78	84	98	100	---	26	58	80	100	---	100	---	---	---	---	---	---	---	---	---	---	
Vavet 447.....	0	0	2	98	98	98	96	96	96	96	96	96	96	96	96	96	---	---	---	---	---	---	---	---	---	
Trebi 448.....	0	0	2	0	2	0	2	96	96	96	0	8	10	66	98	100	---	---	---	2	10	100	---	---	---	
Festland 452.....	0	0	0	92	96	96	96	96	96	90	98	98	96	98	98	98	98	98	100	---	---	---	---	---	---	
Minsturd 439.....	0	0	2	34	96	96	100	---	---	4	70	92	98	98	98	98	92	100	---	86	98	98	96	96	98	98

<sup>1</sup> Begun July 30, ended Aug. 6.  
<sup>2</sup> Begun Aug. 22, ended Aug. 29.  
<sup>3</sup> Begun Sept. 18, ended Sept. 23.

<sup>4</sup> Begun Aug. 5, ended Aug. 12.  
<sup>5</sup> Begun Sept. 3, ended Sept. 10.  
<sup>6</sup> Begun Sept. 23, ended Sept. 30.

<sup>7</sup> Begun Aug 15, ended Aug 22.  
<sup>8</sup> Begun Sept. 9, ended Sept. 16.  
<sup>9</sup> Begun Sept. 30 ended Oct. 7

TABLE 3 —Percentages of germination of barley varieties harvested at different stages of maturity and stored at different temperatures, 1931

[100 seeds collected for each test]

HARD-DOUGH STAGE, COLLECTED JULY 11<sup>1</sup>

Storage temperature and variety	Germination in first test after storage for indicated number of days			Germination in second test after storage for indicated number of days			Germination in third test after storage for indicated number of days			Germination in fourth test after storage for indicated number of days			Germination in fifth test after storage for indicated number of days			Germination in sixth test after storage for indicated number of days		
	3	5	7	3	5	7	3	5	7	3	5	7	3	5	7	3	5	7
0°C.:																		
Peatland 452.....				1	1	3	4	13	42	5	16	48	40	44	51	66	78	81
Trebi 448.....				0	0	3	0	0	31	1	2	19	0	1	13	0	3	6
Manchuria 184.....				1	2	4	3	3	5	5	6	9	7	7	8	6	7	8
Glabron 445.....				0	0	1	4	6	12	0	0	0	0	0	2	0	3	5
10°C.:																		
Peatland 452.....				2	5	8	22	34	45	45	59	67	63	67	71	69	74	77
Trebi 448.....				2	3	4	2	11	23	5	15	40	7	9	22	4	12	23
Manchuria 184.....				5	12	17	14	24	31	27	31	38	31	33	37	26	32	34
Glabron 445.....				2	4	7	1	5	10	4	6	20	6	9	15	1	7	9
20°C.:																		
Peatland 452.....	0	0	1	12	18	23	46	50	55	78	86	88	95	96	97	100	-----	-----
Trebi 448.....	1	1	2	2	2	2	2	9	15	1	6	7	9	13	17	9	15	17
Manchuria 184.....	1	4	5	10	14	14	23	24	26	27	27	29	31	31	33	36	41	48
Glabron 445.....	3	4	10	0	0	0	1	1	1	0	2	2	2	2	4	5	5	9
30°C.:																		
Peatland 452.....				40	97	97	100	-----	-----	99	99	99	98	98	98	100	-----	-----
Trebi 448.....				0	0	2	0	0	6	0	2	6	0	6	12	2	88	95
Manchuria 184.....				11	23	31	64	88	96	98	100	-----	98	98	98	99	100	-----
Glabron 445.....				13	25	41	79	96	97	91	97	97	99	99	99	99	99	99
35°C.:																		
Peatland 452.....				82	99	100	100	-----	-----	100	-----	-----	98	100	-----	100	-----	-----
Trebi 448.....				2	7	11	7	7	9	9	15	24	42	63	80	26	96	98
Manchuria 184.....				21	33	48	95	100	-----	97	99	99	99	99	99	100	-----	-----
Glabron 445.....				1	4	17	76	93	96	85	99	99	99	99	99	99	99	99

RIPE STAGE, COLLECTED JULY 20<sup>2</sup>

0°C.:																		
Peatland 452.....				90	93	93	97	98	98	93	93	94	99	99	99	100	-----	-----
Trebi 448.....				0	1	1	0	0	0	0	0	0	0	0	3	0	1	2
Manchuria 184.....				22	32	50	21	24	34	29	42	54	36	44	57	27	54	68
Glabron 445.....				5	19	39	0	11	31	1	14	36	3	10	28	12	33	48
10°C.:																		
Peatland 452.....				92	94	94	92	95	96	94	94	95	96	96	96	100	-----	-----
Trebi 448.....				1	2	4	0	0	1	0	0	0	1	1	2	0	1	2
Manchuria 184.....				16	35	53	13	20	30	27	35	50	38	46	57	67	82	87
Glabron 445.....				6	16	34	13	19	25	14	35	54	22	40	55	33	55	66
20°C.:																		
Peatland 452.....	86	96	98	100	-----	-----	100	-----	-----	100	-----	-----	100	-----	-----	100	-----	-----
Trebi 448.....	0	0	2	0	0	1	0	1	2	0	0	4	0	1	3	1	6	11
Manchuria 184.....	13	31	43	19	41	57	14	29	48	83	92	95	98	98	98	98	98	98
Glabron 445.....	3	10	29	3	32	47	13	34	51	55	77	84	83	89	93	98	98	98
30°C.:																		
Peatland 452.....				99	100	-----	98	98	98	99	99	99	100	-----	-----	100	-----	-----
Trebi 448.....				0	0	1	0	3	8	9	77	88	36	93	100	66	100	-----
Manchuria 184.....				87	100	-----	100	-----	-----	99	100	-----	100	-----	-----	100	-----	-----
Glabron 445.....				82	96	98	97	98	99	99	99	99	98	98	98	97	97	97
35°C.:																		
Peatland 452.....				99	99	99	100	-----	-----	100	-----	-----	100	-----	-----	100	-----	-----
Trebi 448.....				0	3	7	0	8	18	8	79	97	33	99	99	88	98	98
Manchuria 184.....				96	98	99	100	-----	-----	99	-----	-----	99	100	-----	100	-----	-----
Glabron 445.....				86	94	95	91	94	95	100	-----	-----	97	98	99	99	100	-----

<sup>1</sup> The first test was made July 11, second test July 18, third test July 25, fourth test Aug. 1, fifth test Aug. 8, sixth test Aug. 15.<sup>2</sup> The first test was made July 20, second test July 28, third test Aug. 3, fourth test Aug. 10, fifth test Aug. 17, sixth test Aug. 24.

TABLE 4.—Percentages of germination of rye varieties harvested at different stages of maturity and stored at different temperatures, 1929

[100 seeds taken for each test]

Storage temperature and variety	Germination at soft-dough stage, collected July 2, after storage for indicated number of days						Germination at hard-dough stage, collected July 6, after storage for indicated number of days						Germination at ripe stage, collected July 10, after storage for indicated number of days					
	First test <sup>1</sup>			Second test <sup>2</sup>			First test <sup>1</sup>			Second test <sup>2</sup>			First test <sup>1</sup>			Second test <sup>2</sup>		
	3	5	7	3	7		3	5	7	3	7		3	5	7	3	7	
0° C :																		
Green select 102.....	47	57	66	52	66	53	71	83	79	87	84	98	64	70	75	94	96	
Rosen 82.....	76	81	85	12	27	86	90	92	94	98	76	81	85	84	100			
Prolific 89.....	72	83	86	12	26	13	20	29	30	39								
Synthetic 92.....	40	43	51	70	78	89	90	94	93	98	72	79	84	95	99			
Emerald 92.....	41	53	63	48	62	85	90	91	90	96	84	91	95	100				
Colorless 104.....	47	55	64	48	61	62	72	76	89	93	76	83	89	79	90			
Swedish 2.....	65	79	86	74	83	67	86	89	94	99	78	92	93	98	99			
Dakold 93.....	76	78	82	83	89	84	94		95	98	77	85	87	86	99			
Green select 103.....	20	26	36	15	25	87	94	96	66	87	45	60	87	90	92			
Synthetic 90.....	28	34	42	32	47	87	92	93	97	99	67	82	86	95	98			
Synthetic 91.....	75	76	78	69	76	87	91	92	94	94	98							
10° C :																		
Green select 102.....	59	70	73	77	83	84	85	87	98	98	93	97	99	98	98			
Rosen 82.....	11	29	34	47	79	92	93	95	92	94	88	95	96	100				
Prolific 89.....	3	8	13	20	27	3		4	8	15								
Synthetic 92.....	27	33	42	49	66	96	99		100		95				100			
Emerald 92.....	39	49	62	68	85	99			100		97	98			99	99		
Colorless 104.....	43	56	62	77	89	91	95	96	95	97	85	95	96	96	97			
Swedish 2.....	55	67	73	87	92	100			100		100				99	99		
Dakold 93.....	75	81	86	96	98	95	100		97	98	98	100			95	97		
Green select 103.....	15	30	40	53	75	91	93		95	95	97	99			100			
Synthetic 90.....	27	36	47	69	83	99			98	99	96	97			100			
Synthetic 91.....	36	48	62	80	92	99			100		99				100	100		
20° C :																		
Green select 102.....				90	92	94	96		92	92	86	92	93	97	97			
Rosen 82.....	66	87	91	87	87	78	92	94	100		97	98			97			
Prolific 89.....	87	92	94	99	99	84	61	67	86	91								
Synthetic 92.....	78	94	95	97	97	87	93	96	99	99	90	97			99	100		
Emerald 92.....	87	95	96	95	98	96	98	99	100		97	99			99	99		
Colorless 104.....	80	94		92	94	89	95		99	99	86	92	93	98	98			
Swedish 2.....	91	97	98	99	99	95	97	98	100		97	99			97	98		
Dakold 93.....	93	98		98	99	96	99		99	99	89	94	96	100				
Green select 103.....	76	88	93	93	96	97	98	99	98	98	93	97	97	100				
Synthetic 90.....	60	77	83	99	99	85	91	92			89	98	98	99	97			
Synthetic 91.....	76	83	84	93	93	96			100		98	98	98	100				

<sup>1</sup> Begun Aug. 14, ended Aug. 21.<sup>2</sup> Begun Sept. 10, ended Sept 17.

TABLE 5.—Percentages of germination of rye varieties harvested at different stages of maturity and stored at different temperatures, 1930  
[50 seeds taken for each test]

Storage temperature and variety	Germination at soft-dough stage, collected June 28, after storage for indicated number of days									Germination at hard-dough stage, collected July 2, after storage for indicated number of days									Germination at ripe stage, collected July 11, after storage for indicated number of days								
	First test 1			Second test 2			Third test 3			First test 4			Second test 5			Third test 6			First test 7			Second test 8			Third test 9		
	3	5	7	3	5	7	3	5	7	3	5	7	3	5	7	3	5	7	3	5	7	3	5	7	3	5	7
0° C.:																											
Green Select 102	4	10	16	0	2	14	0	6	18	8	14	14	12	22	22	6	12	32	54	66	80	88	96	100	100	100	100
Rosen 82	6	28	36	0	6	14	0	6	18	0	4	12	4	6	10	0	16	28	84	88	94	96	96	96	96	96	96
Prolific 89	2	14	20	0	4	18	0	4	10	0	12	12	0	4	8	0	2	16	64	68	68	92	96	96	98	98	
Synthetic 92	8	22	42	0	2	10	2	2	28	18	22	34	38	48	52	36	38	44	70	78	82	94	94	94	96	96	
Emerald 92	0	8	20	0	8	30	0	4	32	16	22	34	26	38	44	30	34	54	80	84	90	90	90	94	94	94	
Colorless 104	16	34	40	0	8	16	0	8	30	2	2	12	12	18	22	4	10	30	66	68	76	84	86	92	94	94	
Swedish 2	6	14	26	2	14	28	2	10	24	20	28	42	40	44	52	36	40	54	94	98	98	98	98	98	98	98	
Dakota 93	16	34	46	6	20	36	8	16	44	70	70	76	72	86	88	82	84	96	100	100	100	100	100	100	100	100	
Montagney	50	70	78	12	60	82	10	58	80	74	84	92	78	92	98	76	88	96	86	86	86	86	86	86	86	86	
Green Select 103	10	22	34	0	2	20	0	12	28	14	18	26	22	30	36	12	22	42	80	86	86	86	86	86	86	86	
Synthetic 90	4	14	16	0	2	20	0	10	44	44	50	42	48	50	52	28	36	50	72	78	80	86	86	86	86	86	
Synthetic 91	12	32	40	0	16	2	14	36	34	36	40	52	20	22	48	48	56	68	76	76	84	96	96	96	96	96	
7° C.:																											
Green Select 102	2	8	16	0	2	14	0	0	2	2	4	18	16	26	28	48	60	70	44	50	72	94	94	94	100	100	
Rosen 82	10	12	16	2	2	4	2	2	6	2	2	6	28	42	48	64	76	82	76	78	90	94	94	100	100	100	
Prolific 89	0	2	4	0	4	6	0	8	16	0	0	0	0	10	44	54	60	68	70	80	90	90	90	90	90	90	
Synthetic 92	0	4	4	0	2	4	0	4	20	18	22	26	70	78	82	92	92	80	82	82	92	92	100	100	100	100	
Emerald 92	2	4	10	6	8	18	2	8	26	8	10	30	60	68	70	68	80	86	80	84	92	96	96	96	96	96	
Colorless 104	0	4	6	0	4	6	0	4	24	2	2	10	14	24	32	74	78	82	64	82	90	92	98	94	100	100	
Swedish 2	0	4	16	0	4	6	0	4	24	4	4	30	64	6	12	30	46	54	94	96	86	88	88	100	98	98	
Dakota 93	12	22	26	10	24	34	56	68	78	68	82	92	92	100	92	94	94	88	98	98	98	98	100	100	100	100	
Montagney	34	58	70	30	56	74	56	68	78	68	82	92	92	100	92	94	94	88	98	98	98	98	100	100	100	100	
Green Select 103	2	2	6	0	10	14	0	14	34	12	14	22	30	58	64	58	76	84	84	88	94	98	98	100	100	100	
Synthetic 90	6	6	16	16	16	18	24	28	36	24	38	46	58	68	88	92	92	88	88	90	96	96	98	100	100	100	
Synthetic 91	6	10	16	0	8	12	8	20	46	24	38	46	58	68	88	92	92	100	88	90	96	96	98	100	100	100	
14° C.:																											
Green Select 102	2	2	6	10	20	32	68	82	84	0	8	14	98	98	98	98	100	38	44	56	96	96	96	96	98	98	
Rosen 82	12	16	24	88	92	96	98	98	98	4	12	30	94	94	94	96	96	68	76	82	98	98	98	96	96	96	
Prolific 89	0	10	22	38	50	62	82	82	82	2	6	14	98	98	98	98	96	32	50	64	100	100	100	100	98	98	
Synthetic 92	54	56	62	62	68	76	88	100	100	32	42	58	96	100	100	96	98	100	74	82	100	100	100	100	98	98	
Emerald 92	4	8	8	46	48	50	78	92	94	16	38	48	92	92	92	94	96	98	80	86	92	100	100	100	100	100	
Colorless 104	14	24	32	80	82	86	84	84	94	12	40	50	100	100	100	94	96	98	78	86	92	100	100	100	100	100	



Swedish 2	12	30	40	38	94	94	86	50	92	28	42	58	100	---	---	---	86	88	96	100	---	---	---	100
Dakota 93	26	86	50	88	96	96	100	---	---	---	---	---	---	---	---	---	98	100	82	88	90	---	---	---
Montgomery	84	30	58	88	98	98	98	98	98	96	98	98	100	---	---	---	98	100	82	88	90	---	---	---
Green Select 103	4	6	10	84	90	92	100	---	---	---	---	---	---	---	---	---	78	84	86	100	---	---	---	---
Synthetic 90	0	0	3	38	46	48	32	38	32	38	32	38	98	98	98	98	92	90	70	74	92	100	---	---
Synthetic 91	14	22	42	54	58	64	92	96	96	22	34	50	100	---	---	---	56	72	78	94	94	96	---	---

<sup>1</sup> Begun July 28, ended Aug. 4.  
<sup>2</sup> Begun Aug. 4, ended Aug. 11.  
<sup>3</sup> Begun Aug. 11, ended Aug. 18.  
<sup>4</sup> Begun Aug. 18, ended Aug. 28.  
<sup>5</sup> Begun Aug. 28, ended Sept. 7.  
<sup>6</sup> Begun Sept. 7, ended Sept. 20.  
<sup>7</sup> Begun Sept. 20, ended Sept. 29.  
<sup>8</sup> Begun Sept. 29, ended Oct. 7.  
<sup>9</sup> Begun Oct. 7, ended Oct. 15.  
<sup>10</sup> Begun Oct. 15, ended Oct. 22.  
<sup>11</sup> Begun Oct. 22, ended Oct. 29.  
<sup>12</sup> Begun Oct. 29, ended Nov. 6.  
<sup>13</sup> Begun Nov. 6, ended Nov. 13.  
<sup>14</sup> Begun Nov. 13, ended Nov. 20.  
<sup>15</sup> Begun Nov. 20, ended Nov. 27.  
<sup>16</sup> Begun Nov. 27, ended Dec. 4.  
<sup>17</sup> Begun Dec. 4, ended Dec. 11.  
<sup>18</sup> Begun Dec. 11, ended Dec. 18.  
<sup>19</sup> Begun Dec. 18, ended Dec. 25.  
<sup>20</sup> Begun Dec. 25, ended Jan. 1, 1900.

TABLE 6.—*Germination percentages of rye varieties harvested at different stages of maturity and stored at different temperatures, 1931*

[100 seeds collected for each test]

HARD-DOUGH STAGE, COLLECTED JULY 6<sup>1</sup>

Storage temperature and variety	Germination in first test after storage for indicated number of days			Germination in second test after storage for indicated number of days			Germination in third test after storage for indicated number of days			Germination in fourth test after storage for indicated number of days			Germination in fifth test after storage for indicated number of days			Germination in sixth test after storage for indicated number of days		
	3	5	7	3	5	7	3	5	7	3	5	7	3	5	7	3	5	7
0° C.:																		
Green Select 102.	50	61	63	73	90	94	79	90	93	79	91	96	80	90	95	87	97	98
Rosen 82.	21	30	39	78	88	91	78	86	88	82	91	96	67	78	94	85	95	98
Prolific 89 <sup>2</sup> .	---	---	---	1	5	22	2	14	23	5	14	21	9	17	33	9	17	35
Emerald 92.	73	83	86	92	96	96	80	86	91	86	95	96	90	98	98	95	97	97
10° C.:																		
Green Select 102.	54	75	77	43	68	79	84	93	96	92	97	98	81	95	97	95	97	98
Rosen 82.	26	45	56	70	85	91	65	84	89	88	93	95	82	92	95	96	100	---
Prolific 89 <sup>2</sup> .	---	---	---	1	5	9	4	10	25	0	5	22	21	26	39	18	31	45
Emerald 92.	76	83	88	83	88	88	92	95	96	96	98	99	94	98	99	100	---	---
20° C.:																		
Green Select 102.	47	69	76	30	60	63	43	70	74	87	94	95	88	96	99	98	100	---
Rosen 82.	20	34	49	19	40	46	34	73	81	81	90	95	79	92	96	89	96	97
Prolific 89 <sup>2</sup> .	2	4	25	2	19	26	6	28	45	14	23	33	60	72	79	84	92	93
Emerald 92.	75	86	87	75	87	89	82	92	96	95	98	99	100	---	---	100	---	---
30° C.:																		
Green Select 102.	59	76	86	34	68	71	29	83	87	88	99	100	90	99	100	100	---	---
Rosen 82.	25	45	53	49	78	86	42	86	91	96	99	99	93	99	99	100	---	---
Prolific 89 <sup>2</sup> .	---	---	---	2	8	28	13	39	73	61	72	84	98	100	---	100	---	---
Emerald 92.	77	86	89	45	60	73	74	93	94	100	---	---	100	---	---	100	---	---
35° C.:																		
Green Select 102.	52	80	87	14	50	59	66	88	92	92	98	98	99	100	---	100	---	---
Rosen 82.	22	45	54	28	40	49	64	82	84	90	92	94	97	98	98	100	---	---
Prolific 89 <sup>2</sup> .	---	---	---	5	21	42	41	71	86	81	94	96	97	98	98	100	---	---
Emerald 92.	80	89	90	62	78	87	88	94	96	99	100	---	98	98	98	100	---	---

RIPE STAGE, COLLECTED JULY 20<sup>3</sup>

Storage temperature and variety	Germination in first test after storage for indicated number of days			Germination in second test after storage for indicated number of days			Germination in third test after storage for indicated number of days			Germination in fourth test after storage for indicated number of days			Germination in fifth test after storage for indicated number of days			Germination in sixth test after storage for indicated number of days		
	3	5	7	3	5	7	3	5	7	3	5	7	3	5	7	3	5	7
0° C.:																		
Green Select 102.	---	---	---	65	82	93	76	94	97	100	---	---	98	99	99	100	---	---
Rosen 82.	---	---	---	59	80	86	68	80	94	97	98	98	96	97	97	98	98	98
Prolific 89.	---	---	---	28	56	69	50	61	78	76	87	90	88	96	98	88	94	96
Emerald 92.	---	---	---	93	99	99	97	98	99	98	99	9	99	99	99	96	97	98
10° C.:																		
Green Select 102.	---	---	---	70	88	91	80	93	95	100	---	---	100	---	---	99	99	99
Rosen 82.	---	---	---	68	86	94	74	87	94	100	---	---	99	99	99	100	---	---
Prolific 89.	---	---	---	23	65	74	76	88	91	94	97	98	97	100	---	99	100	---
Emerald 92.	---	---	---	89	96	99	99	99	99	98	98	98	99	99	99	100	---	---
20° C.:																		
Green Select 102.	56	77	87	82	91	93	94	98	98	100	---	---	100	---	---	100	---	---
Rosen 82.	40	53	72	47	76	79	81	92	93	94	95	96	98	98	98	99	99	99
Prolific 89.	9	25	50	33	64	75	49	82	90	94	95	98	100	---	---	100	---	---
Emerald 92.	72	91	95	99	100	---	100	---	100	---	---	---	100	---	---	100	---	---
30° C.:																		
Green Select 102.	---	---	---	72	93	94	96	100	---	100	---	---	99	99	99	100	---	---
Rosen 82.	---	---	---	71	87	88	96	100	---	100	---	---	99	99	99	100	---	---
Prolific 89.	---	---	---	25	66	83	45	81	94	100	---	---	100	---	---	98	99	99
Emerald 92.	---	---	---	87	99	99	99	99	99	100	---	---	99	99	99	100	---	---
35° C.:																		
Green Select 102.	---	---	---	76	90	94	82	97	97	100	---	---	100	---	---	99	99	99
Rosen 82.	---	---	---	53	78	77	81	91	95	97	97	97	96	98	98	99	99	99
Prolific 89.	---	---	---	15	46	66	78	90	94	100	---	---	100	---	---	100	---	---
Emerald 92.	---	---	---	94	98	98	100	---	---	100	---	---	99	99	99	100	---	---

<sup>1</sup> The first test was made July 8, second test July 15, third test July 22, fourth test July 29, fifth test Aug. 5, and sixth test Aug. 12.<sup>2</sup> Collected July 11. Spring variety.<sup>3</sup> The first test was made July 20, second test July 28, third test Aug. 3, fourth test Aug. 10, fifth test Aug. 17, and sixth test Aug. 24.

TABLE 7.—Percentages of germination of early and late oat varieties harvested at different stages of maturity and stored at different temperatures, 1929

[100 seeds taken for each test]

## EARLY VARIETIES

Storage temperature and variety	Germination at soft-dough stage <sup>1</sup> after storage for indicated number of days						Germination at hard-dough stage <sup>2</sup> after storage for indicated number of days						Germination at ripe stage <sup>3</sup> after storage for indicated number of days					
	First test <sup>4</sup>		Second test <sup>5</sup>		Third test <sup>6</sup>		First test <sup>4</sup>		Second test <sup>5</sup>		Third test <sup>6</sup>		First test <sup>4</sup>		Second test <sup>5</sup>		Third test <sup>6</sup>	
	5	7	5	7	5	7	5	7	5	7	5	7	5	7	5	7	5	7
	5	7	5	7	5	7	5	7	5	7	5	7	5	7	5	7	5	7
0° C																		
Double Cross 730.....																		
Minrus 693.....																		
Rainbow 710.....																		
Gopher 674.....	29	62	60	88	85	93							78	90	71	93		
North Dakota 22005.....	11	21	0	16	45	58	2	23	4	39	66	75	0	12	0	40	93	95
Iogold 711.....	0	15	5	36	62	84							1	9	2	12	73	87
Liberty Hull less 676.....	27	42	51	69	58	66							52	64	41	68	99	
10° C																		
Double Cross 730.....																		
Minrus 693.....																		
Rainbow 710.....																		
Gopher 674.....	20	45	39	59	72	77							7	47	47	77		
North Dakota 22005.....	0	0	0	12			0	10	10	48			9	36	10	74	100	
Iogold 711.....	1	3	0	8	57	65							2	19		11	44	
Liberty Hull-less 676.....	10	24	39	68											75	88	62	81
20° C																		
Double Cross 730.....																		
Minrus 693.....																		
Rainbow 710.....																		
Gopher 674.....	21	67	63	64	84								70	94	95	95		
North Dakota 22005.....	21	50	89	95			8	29	91	99			16	52	99	100		
Iogold 711.....	13	25	49	66	77	79							47	70	80	84		
Liberty Hull-less 676.....	65	89	98										95	98	100			

## LATE VARIETIES

0° C																		
Anthony 686.....	0	13	2	20	61	77	0	19	3	13	39	76	6	30	0	6	63	79
Green Russian 713.....							5	24	32	63	49	70	22	47	37	62	98	
Green Russian 677.....	33	59	63	79			27	60	59	84			44	56	56	71	95	99
White Russian × Victory 696.....																		
Minota 512.....	2	16	4	37	9	41	3	20	5	41	88	92	9	22	1	31	85	90
Victory 514.....	8	24	1	28	6	28	5	29	1	20	65	84	14	55	20	54	77	83
North Dakota 20014.....	1	2	0	3	84	91	2	12	1	5	39	58	1	2	0	3	84	94
10° C																		
Anthony 686.....	0	5	0	5			0	5	0	5			4	15	5	19	94	95
Green Russian 713.....	2	14	0	46			36	70	6	27	82	86	27	65	62	80		
Green Russian 677.....	7	9	6	23			20	70	17	55			38	68	38	80		
White Russian × Victory 696.....																		
Minota 512.....	0	3	0	5			0	8	0	19			2	4	11	43	99	
Victory 514.....	1	30	1	37			2	21	6	29			12	72	26	70	98	
North Dakota 20014.....	0	2	2	8			0	0	0	0	58	66	0	1	12	27		
20° C																		
Anthony 686.....	36	53	96	97			3	7	48	83			1	11	73	76		
Green Russian 713.....	38	78	89	94			7	68	65	89			43	82	95	97		
Green Russian 677.....	42	55	59	61			45	97	76	79			53	66	94	96		
White Russian × Victory 696.....																		
Minota 512.....	22	70	92	99			13	42	90	97			15	44	94	97		
Victory 514.....	32	70	92	94			10	54	65	98			21	89	86	91		
North Dakota 20014.....	0	10	40	43	92		0	9	41	65	98		0	17	93	97		

<sup>1</sup> Early varieties collected July 18; late varieties July 24<sup>2</sup> Early varieties collected July 23; late varieties July 29.<sup>3</sup> Early varieties collected July 26; late varieties Aug. 1.<sup>4</sup> Begun Aug. 7; ended Aug. 14.<sup>5</sup> Begun Aug. 30; ended Sept. 6.<sup>6</sup> Begun Sept. 23; ended Sept. 30.





TABLE 9.—Germination percentages of early oat varieties harvested at different dates and stored at different temperatures, 1931

[100 seeds taken for each test]

HARD-DOUGH STAGE, COLLECTED JULY 11<sup>1</sup>

Storage temperature and variety	Germination in first test after storage for indicated number of days			Germination in second test after storage for indicated number of days			Germination in third test after storage for indicated number of days			Germination in fourth test after storage for indicated number of days			Germination in fifth test after storage for indicated number of days			Germination in sixth test after storage for indicated number of days		
	3	5	7	3	5	7	3	5	7	3	5	7	3	5	7	3	5	7
0° C.																		
Double Cross 730...				1	2	3	0	4	27	1	5	32	1	2	6	1	7	22
Minrus 693...				0	2	18	0	0	21	1	2	26	0	1	15	0	6	25
Gopher 674 <sup>2</sup> ...				40	89	92	14	72	81	30	78	86	76	79	84	57	89	92
Logold 711 <sup>3</sup> ...				0	0	2	0	4	39	1	2	46	6	14	36	6	46	55
10° C.																		
Double Cross 730...				0	4	5	0	0	5	2	12	35	3	8	16	5	36	49
Minrus 693...				0	4	11	0	3	9	5	36	53	24	30	58	8	79	81
Gopher 674 <sup>2</sup> ...				40	80	90	31	80	91	55	90	93	87	95	99	56	95	96
Logold 711 <sup>3</sup> ...				0	2	10	1	8	33	3	46	48	38	54	56	44	77	81
20° C.																		
Double Cross 730...	6	10	21	2	8	17	5	28	45	57	74	87	68	73	85	94	99	99
Minrus 693...	8	25	47	7	42	61	6	53	70	28	67	76	76	85	90	55	93	94
Gopher 674 <sup>2</sup> ...	48	82	86	60	90	98	85	91	93	88	93	93	97	98	98	95	99	99
Logold 711 <sup>3</sup> ...	0	7	24	0	20	43	15	53	69	73	88	89	87	91	96	88	98	98
30° C.																		
Double Cross 730...				20	69	74	74	95	99	89	97	99	100	---	---	92	98	98
Minrus 693...				21	54	69	25	88	91	53	87	94	96	96	97	72	97	97
Gopher 674 <sup>2</sup> ...				42	84	93	92	98	99	93	98	98	100	---	---	96	98	99
Logold 711 <sup>3</sup> ...				52	68	79	73	89	94	83	97	99	98	98	98	84	99	100
35° C.																		
Double Cross 730...				13	52	61	81	89	93	94	100	---	100	---	---	98	100	---
Minrus 693...				15	36	49	23	84	91	79	99	99	94	99	99	64	92	95
Gopher 674 <sup>2</sup> ...				40	75	80	83	93	95	92	100	---	100	---	---	92	98	98
Logold 711 <sup>3</sup> ...				68	89	95	69	95	98	91	97	100	99	100	---	91	99	99

RIPE STAGE, COLLECTED JULY 22<sup>3</sup>

0° C.																		
Double Cross 730...				34	67	68	12	55	77	23	75	81	43	88	94	77	94	96
Minrus 693...				7	56	63	4	52	77	16	77	86	28	83	90	69	90	93
Gopher 674 <sup>2</sup> ...				96	99	99	81	96	98	93	98	98	93	95	96	92	97	97
Logold 711 <sup>3</sup> ...				42	73	79	39	84	91	69	93	93	88	99	99	88	95	95
10° C.																		
Double Cross 730...				28	51	54	5	44	55	31	86	90	62	90	93	92	96	96
Minrus 693...				16	53	68	2	38	67	19	83	87	41	90	90	83	99	99
Gopher 674 <sup>2</sup> ...				84	86	88	83	95	95	96	98	99	90	95	96	88	92	92
Logold 711 <sup>3</sup> ...				42	74	80	15	72	80	82	98	98	88	96	96	92	95	96
20° C.																		
Double Cross 730...				46	78	80	74	92	93	89	99	100	98	99	99	100	---	---
Minrus 693...	1	21	25	29	79	82	32	92	96	63	100	---	91	100	---	93	95	95
Gopher 674 <sup>2</sup> ...	74	97	99	2	17	22	98	99	99	97	98	98	100	---	---	93	98	99
Logold 711 <sup>3</sup> ...	1	40	50	0	2	5	90	96	96	90	94	94	83	99	99	96	97	97
30° C.																		
Double Cross 730...				59	96	96	95	95	98	100	---	---	98	100	---	100	---	---
Minrus 693...				72	99	99	73	100	---	90	100	---	95	99	99	86	98	98
Gopher 674 <sup>2</sup> ...				100	---	---	96	99	100	98	99	99	97	97	97	98	100	---
Logold 711 <sup>3</sup> ...				87	96	96	97	99	99	100	---	---	100	---	---	96	99	99
35° C.																		
Double Cross 730...				98	97	97	99	99	99	98	100	---	99	100	---	100	---	---
Minrus 693...				76	93	95	94	98	99	96	99	99	95	100	---	92	98	98
Gopher 674 <sup>2</sup> ...				100	---	---	96	97	98	93	100	---	94	97	97	90	96	97
Logold 711 <sup>3</sup> ...				88	98	98	97	100	---	99	100	---	99	100	---	96	99	99

<sup>1</sup> The first test was made July 11, second test July 18, third test July 25, fourth test Aug. 1, fifth test Aug. 8, sixth test Aug. 15.<sup>2</sup> Collected July 8.<sup>3</sup> The first test was made July 22, second test July 30, third test Aug. 5, fourth test Aug. 12, fifth test Aug. 19, and sixth test Aug. 26.

TABLE 10.—*Germination percentages of late oat varieties harvested at different dates and stored at different temperatures, 1931*

[100 seeds taken for each test]

HARD-DOUGH STAGE, COLLECTED JULY 13<sup>1</sup>

Storage temperature and variety	Germination in first test after storage for indicated number of days			Germination in second test after storage for indicated number of days			Germination in third test after storage for indicated number of days			Germination in fourth test after storage for indicated number of days			Germination in fifth test after storage for indicated number of days			Germination in sixth test after storage for indicated number of days		
	3	5	7	3	5	7	3	5	7	3	5	7	3	5	7	3	5	7
0° C.																		
Anthony 686.....				0	0	1	0	1	20	0	0	2	0	2	11	0	2	7
Russian Green.....																		
713.....				0	15	24	1	42	55	0	6	30	0	5	39	0	13	55
Victory 514.....				0	7	17	0	21	42	0	0	14	0	2	7	0	3	21
North Dakota 20014.....				0	0	2	0	0	1	0	0	0	0	0	1	9	9	16
10° C.																		
Anthony 686.....				0	0	0	0	5	7	1	4	10	1	12	28	0	40	58
Russian Green.....																		
713.....				1	4	6	2	28	62	2	31	46	3	41	52	15	77	87
Victory 514.....				0	0	0	0	8	22	0	4	20	1	8	26	9	48	60
North Dakota 20014.....				0	0	0	0	0	8	0	1	3	1	10	21	6	49	57
20° C.																		
Anthony 686.....	0	2	16	0	0	0	0	7	26	0	9	35	1	38	65	24	80	89
Russian Green.....																		
713.....	0	4	23	4	20	26	6	43	71	5	59	73	12	68	76	44	89	92
Victory 514.....	0	4	18	0	2	14	0	11	36	1	28	42	34	77	83	66	95	98
North Dakota 20014.....	0	0	1	0	0	0	0	0	5	0	7	21	2	38	46	41	83	89
30° C.																		
Anthony 686.....				14	70	81	43	79	85	63	86	86	95	99	99	96	99	99
Russian Green.....																		
713.....				27	75	83	77	94	94	77	94	95	88	100	-----	88	98	99
Victory 514.....				0	14	51	45	82	88	50	55	56	96	99	99	95	99	99
North Dakota 20014.....				0	6	13	23	70	79	71	89	90	94	99	99	92	100	-----
35° C.																		
Anthony 686.....				12	74	76	78	89	90	73	91	91	78	97	97	96	100	-----
Russian Green.....																		
713.....				13	69	78	50	96	98	73	97	97	66	100	-----	84	97	98
Victory 514.....				8	50	60	67	91	93	53	91	93	78	91	92	77	97	97
North Dakota 20014.....				16	37	50	64	86	87	64	92	95	81	92	93	94	99	99

<sup>1</sup> The first test was made July 13, second test July 20, third test July 28, fourth test Aug. 3, fifth test Aug. 10, and sixth test Aug. 17.

TABLE 10.—*Germination percentages of late oat varieties harvested at different dates and stored at different temperatures, 1931—Continued*

[100 seeds taken for each test]

RIPE STAGE, COLLECTED JULY 22<sup>2</sup>

Storage temperature and variety	Germination in first test after storage for indicated number of days			Germination in second test after storage for indicated number of days			Germination in third test after storage for indicated number of days			Germination in fourth test after storage for indicated number of days			Germination in fifth test after storage for indicated number of days			Germination in sixth test after storage for indicated number of days		
	3	5	7	3	5	7	3	5	7	3	5	7	3	5	7	3	5	7
0° C.:																		
Anthony 686.....				0	6	13	0	5	29	9	59	67	4	56	74	30	86	91
Russian Green.....																		
713.....				6	38	48	0	22	67	6	71	80	9	68	87	31	84	88
Victory 514.....				1	4	11	0	8	41	3	63	75	5	66	79	11	83	83
North Dakota.....																		
20014.....				0	0	3	0	2	7	0	6	19	1	10	34	5	58	72
10° C.:																		
Anthony 686.....				0	25	32	0	36	60	12	73	77	19	80	85	29	93	96
Russian Green.....																		
713.....				3	51	61	4	69	92	8	84	90	37	94	97	80	99	99
Victory 514.....				0	7	15	0	9	34	6	77	84	8	81	88	28	97	99
North Dakota.....																		
20014.....				0	1	3	0	8	17	0	47	60	9	64	74	41	84	86
20° C.:																		
Anthony 686.....	0	9	19	2	37	43	11	81	89	82	95	95	82	99	99	84	94	95
Russian Green.....																		
713.....	1	30	57	5	71	75	12	79	87	77	95	96	77	97	99	98	100	---
Victory 514.....	0	3	11	97	99	99	0	88	62	36	96	97	79	100	---	92	98	98
North Dakota.....																		
20014.....	0	0	0	59	74	81	0	17	58	43	96	96	74	93	94	92	94	96
30° C.:																		
Anthony 686.....				82	87	88	82	97	98	85	88	89	99	100	---	98	100	---
Russian Green.....																		
713.....				75	95	97	80	100	---	97	100	---	97	100	---	98	100	---
Victory 514.....				70	95	96	92	98	98	85	95	95	85	100	---	92	99	99
North Dakota.....																		
20014.....				39	80	85	62	96	97	98	99	99	90	98	99	93	100	---
35° C.:																		
Anthony 686.....				92	95	95	62	92	94	90	97	97	96	98	98	88	98	98
Russian Green.....																		
713.....				74	94	97	85	99	99	95	100	---	100	---	---	98	100	---
Victory 514.....				81	91	91	92	97	97	97	100	---	99	100	---	93	100	---
North Dakota.....																		
20014.....				24	85	95	93	99	100	98	100	---	99	100	---	99	100	---

<sup>2</sup> The first test was made July 22, second test July 30, third test Aug. 5, fourth test Aug. 12, fifth test Aug. 19, and sixth test Aug. 26.



TABLE 11.—Percentages of germination of winter and spring wheat varieties harvested at different stages of maturity and stored at different temperatures, 1929

Storage temperature and variety		[100 seeds taken for each test]									WINTER VARIETIES										
		Germination at soft-dough stage <sup>1</sup> after storage for indicated number of days						Germination at hard-dough stage <sup>2</sup> after storage for indicated number of days						Germination at ripe stage <sup>3</sup> after storage for indicated number of days							
		First test <sup>4</sup>		Second test <sup>5</sup>		Third test <sup>6</sup>		First test <sup>4</sup>		Second test <sup>5</sup>		Third test <sup>6</sup>		First test <sup>4</sup>		Second test <sup>5</sup>		Third test <sup>6</sup>			
		5	7	5	7	5	7	5	7	5	7	5	7	5	7	5	7	5	7		
0° C.:		2	3	0	0	4		0	1	8	0	7	26	11	18	4	7	11	38	15	23
Jobred 1949		2	11	0	25	4		0	15	49	70	36	74	66	86	2	0	3	23	9	29
Kareid 2191		12	33	2	40			60	83	87	95	97	99			58	74	100	100		
Turkey 1488		6	14	19	86			87	95	56	72	51	79			9	13	87	95		
Minturki 1507		15	30	11	49			56	72	0	0	0	9	12	24	27	36	13	50	10	37
Crimean 845		23	38	0	34	4		18	25	15	4	15	57	10	39	20	29	24	67	9	33
Ukraine 2209		11	22	0	42	17		30	63	30	63	8	22	9	27	7	12	13	19	60	85
Ukraine 1506		32	47	8	49	10		18	30	18	30	8	22	9	27	7	12	13	19	60	85
Minbard 1904		0	11	6	73	10		88	18	30	30	8	22	9	27	7	12	13	19	60	85
Red Rock 2154																					
Marquis 1239																					
10° C.:		0	0	2	1	3		2	0	15	27	82	91			11	25	8	90		
Jobred 1949		0	1	2	9	16		2	0	49	70	36	74	66	86	4	9	78	94	84	97
Kareid 2191		1	3	17	46			53	74	96	95	97	99			80	84	94	94		
Turkey 1488		12	29	86	98			90	95	100	100	100	100			88	100	100	100		
Minturki 1507		1	4	55	74	75		18	25	73	18	25	73	84	75	71	77	94	99		
Crimean 845		1	1	3	10			2	27	42	2	27	42	67	75	2	10	87	92	75	98
Ukraine 2209		0	2	1	10	0		5	12	67	5	12	67	84		1	3	48	63	9	32
Ukraine 1506		0	0	29	60			8	12	62	8	12	62	82		60	84	99	99		
Minbard 1904		0	1	14	65			24	38		24	38	89	93		78	86	100	100		
Red Rock 2154		0	1	2	9	100		100	1		1		18	31		17	30	98	98		
Marquis 1239		1	1																		

<sup>1</sup> Winter varieties collected July 5; spring varieties, July 19<sup>2</sup> Winter varieties collected July 11; spring varieties, July 24.<sup>3</sup> Winter varieties collected July 15; spring varieties, July 28<sup>4</sup> Winter varieties, begun Aug 16, ended Aug 23; spring varieties, begun Aug. 22, ended Aug. 29<sup>5</sup> Winter varieties, begun Sept 14, ended Sept 21; spring varieties, begun Sept 16, ended Sept 23<sup>6</sup> Winter varieties, begun Sept. 28, ended Oct. 5; spring varieties, begun Oct 14, ended Oct 21

TABLE 11.—Percentages of germination of winter and spring wheat varieties harvested at different stages of maturity and stored at different temperatures, 1929—Continued

[100 seeds taken for each test]

## WINTER VARIETIES—Continued

Storage temperature and variety	Germination at soft-dough stage after storage for indicated number of days			Germination at hard-dough stage after storage for indicated number of days			Germination at ripe stage after storage for indicated number of days					
	First test		Second test		Third test		First test		Second test		Third test	
	5	7	5	7	5	7	5	7	5	7	5	7
20° C.:												
Iobred 1940.....	2	5	5	26	—	—	3	7	6	45	—	—
Kaared 2191.....	1	1	10	36	—	—	3	7	19	75	—	—
Turkey 1488.....	55	92	93	99	—	—	100	100	98	98	—	—
Minturki 1507.....	98	99	99	99	—	—	98	100	99	99	—	—
Crimean 845.....	42	63	94	98	—	—	76	88	91	95	—	—
Ukranka 2299.....	77	82	70	92	—	—	4	4	27	93	—	—
Minhard 1505.....	3	11	10	57	—	—	9	15	29	68	59	87
Minard 2199.....	95	98	99	99	—	—	28	40	64	78	17	33
Red Rock 2194.....	92	96	77	85	—	—	76	83	97	100	—	—
Marquis 1289.....	0	2	77	85	—	—	4	23	98	98	—	—

## SPRING VARIETIES

Storage temperature and variety	Germination at soft-dough stage after storage for indicated number of days			Germination at hard-dough stage after storage for indicated number of days			Germination at ripe stage after storage for indicated number of days					
	First test		Second test		Third test		First test		Second test		Third test	
	5	7	5	7	5	7	5	7	5	7	5	7
0° C.:												
Nodak 2311.....	0	0	2	23	4	26	2	10	6	31	18	37
Marquis 1289.....	0	11	6	73	10	88	18	30	8	22	9	27
Double Cross 2305.....	—	—	—	—	—	—	0	5	3	15	5	45
Mindum 470.....	0	2	0	2	0	15	2	5	3	32	29	62
Marquette 2202.....	1	2	0	1	0	8	6	6	1	1	6	1
Reward 2204.....	0	0	0	0	7	50	2	2	2	1	11	24
Hope 2207.....	3	19	0	51	11	54	1	3	1	5	30	84
Double Cross 2304.....	—	—	—	—	—	—	0	1	0	22	2	39
Double Cross 2303.....	—	—	—	—	—	—	9	16	3	18	63	91
Kubanka 2310.....	0	16	0	23	3	7	7	16	0	36	1	31
Marquis X Einkorn 2301.....	—	—	—	—	—	—	0	2	1	8	35	69
Supreme 2309.....	19	63	29	91	—	—	31	51	7	87	0	1
Bluestem 169.....	4	20	6	27	0	4	0	0	0	5	0	1
Ceres 2223.....	0	4	2	50	7	55	5	12	12	71	54	95
Reliance 2308.....	3	33	6	28	24	74	2	13	39	81	2	28

10° C.	0	0	3	8	100	100	3	8	18	31	1	17	18	25	64
Nodak 2311	1	1	2	9	100	100	1	1	18	31	17	17	18	25	64
Marquis 1239							0	0	0	7		4	27	45	92
Double Cross 2305							0	0	0	1		2	2	16	37
Mindum 470	1	1	3	4			0	0	1	18		8	11	81	96
Marquillo 2202	0	0	0	0			0	0	4	15		0	0	8	27
Reward 2204	1	2	8	30			0	9	48	70		8	11	81	96
Hope 2297	0	1	1	10			0	0	8	12		0	0	8	27
Double Cross 2304							0	0	2	3					
Double Cross 2303							1	3	3	16		1	3	49	56
Kubanka 2310	3	3	5	9			3	9	0	15		14	53	39	89
Marquis X Enkorn 2301							0	0	0	2		1	3	24	50
Supreme 2309	2	16	22	47			0	4	77	82		61	72	97	99
Bluestem 169	0	0	5	18			0	0	4	5					
Ceres 2223	0	0	23	54			0	3	37	48		12	29	86	97
Reliance 2308	2	2	15	30			1	1	10	16		99	2	13	82
20° C.															
Nodak 2311	0	1	35	71	14	78	0	20	78	91		0	12	55	71
Marquis 1239	0	2	77	85			4	23	97	97		3	10	98	98
Double Cross 2305							0	0	10	20		25	43		
Mindum 470	9	28	31	67	36	76	5	61	48	95		1	27	5	46
Marquillo 2202	15	15	4	7	32	45	0	0	2	20		0	2	33	51
Reward 2204	24	66	86	97			8	48	97	97		4	8	96	100
Hope 2297	2	14	90	93			1	3	61	68		0	0	48	53
Double Cross 2304							0	2	7	12		27	57		81
Double Cross 2303							0	6	65	71		90	93		
Kubanka 2310							7	57	66	91		30	72	69	95
Marquis X Enkorn 2301	0	37	38	57	64	94	3	53	92	97		1	3	85	90
Supreme 2309	34	56	87	91			26	53	95	96		42	52	80	80
Bluestem 169	14	17	52	62	95	95	0	0	95	95					
Ceres 2223	20	58	99	99			8	35	100	100		11	29	94	95
Reliance 2308	2	7	83	85			0	4	94	95		3	13	95	95

TABLE 12.—Percentages of germination of winter and spring wheat varieties harvested at different stages of maturity and stored at different temperatures, 1930

[50 seeds taken for each test]

## WINTER VARIETIES

Storage temperature and variety	Germination at soft-dough stage <sup>1</sup> after storage for indicated number of days									Germination at hard-dough stage <sup>2</sup> after storage for indicated number of days									Germination at ripe stage <sup>3</sup> after storage for indicated number of days								
	First test 4			Second test 5			Third test 6			First test 7			Second test 8			Third test 9			First test 10			Second test 11			Third test 12		
	3	5	7	3	5	7	3	5	7	3	5	7	3	5	7	3	5	7	3	5	7	3	5	7	3	5	7
	0	20	62	0	10	62	0	12	60	0	6	16	10	76	90	62	94	96	66	70	82	92	98	100	98	98	98
0° C.:																											
Minurki 1507.....	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Marquis 1239.....	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Minhard 1505.....	0	10	14	0	0	4	0	8	74	0	0	2	0	54	78	6	74	92	2	2	2	10	44	50	56	80	88
Minard 2109.....	2	18	30	0	8	24	0	8	38	0	0	2	0	24	28	16	50	70	4	4	10	22	42	56	82	88	98
Minard × Minhard 2314.....	2	6	16	0	2	8	0	0	50	0	0	2	0	14	22	18	64	90	0	0	0	16	44	60	66	84	92
Minard × Minhard 2313.....	0	0	12	0	0	16	0	12	62	0	2	2	2	28	42	6	58	90	0	2	20	8	22	38	70	88	96
Minhard × Minurki 2312.....	0	10	38	0	16	56	2	14	52	0	4	4	6	56	66	4	72	96	14	16	30	52	68	72	96	98	100
7° C.:																											
Minurki 1507.....	0	2	4	0	0	18	16	90	98	0	8	24	98	100	100	94	98	98	84	80	88	100	100	100	100	100	100
Marquis 1239.....	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Minhard 1505.....	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Minard 2109.....	0	2	8	0	0	0	0	0	0	0	0	0	18	38	58	20	84	96	4	4	20	48	66	82	98	98	98
Minard × Minhard 2314.....	0	0	0	0	0	0	0	0	0	0	0	0	16	34	54	38	88	92	0	2	30	36	72	78	84	88	96
Minard × Minhard 2313.....	0	0	0	0	0	0	0	0	0	0	0	0	18	44	60	34	88	98	0	2	18	4	38	62	90	94	94
Minhard × Minurki 2312.....	0	0	28	0	0	0	2	26	56	0	4	12	42	86	98	80	96	100	6	8	20	98	98	100	100	100	100
14° C.:																											
Minurki 1507.....	6	20	46	100	100	100	98	100	100	0	12	24	98	98	98	98	98	98	64	64	80	100	100	100	100	100	100
Marquis 1239.....	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Minhard 1505.....	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Minard 2109.....	2	4	24	80	80	82	98	98	98	0	2	22	98	100	100	98	98	98	0	0	20	100	100	100	100	100	100
Minard × Minhard 2314.....	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Minard × Minhard 2313.....	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Minhard × Minurki 2312.....	0	0	2	68	86	88	94	98	98	0	10	20	98	98	98	100	100	100	22	28	56	98	98	100	100	100	100

## SPRING VARIETIES

[illegible]

Winter varieties collected July 7: spring varieties July 8

Winter varieties collected July 9; spring varieties July 14.

Winter varieties collected July 17; spring varieties July 28.

<sup>15</sup> Winter varieties, begun Aug. 21, ended Aug. 28; spring varieties, begun July 28, ended Aug. 5; spring varieties, begun Aug. 21, ended Aug. 28.

Winter varieties, begun Sept. 17, ended Sept. 24; spring varieties, begun Sept. 24, ended Sept. 28.

**Winter varieties, begun Aug. 5, ended Aug. 12; spring vari-**

Winter varieties, begun Sept. 2, ended Sept. 9, spring varieties, begun Sept. 22, ended Sept. 29: spring varieties, begun Sept. 22, ended Sept. 29: spring varieties.

Winter varieties, begun Aug. 14, ended Aug. 21, spring varieties, begun Aug. 27, ended Sept. 29, summer varieties, begun Sept. 27, ended Oct. 29.

11 Winter varieties, begun Sept. 9, ended Sept. 16; spring varieties, begun Sept. 16, ended Oct. 13

Winter varieties, begun Sept. 21, ended Oct. 4; spring var-



TABLE 13.—*Germination percentages of winter wheat varieties harvested at different dates and stored at different temperatures, 1931*

[100 seeds taken for each test]

HARD-DOUGH STAGE, COLLECTED JULY 6<sup>1</sup>

Storage temperature and variety	Germination in first test after storage for indicated number of days			Germination in second test after storage for indicated number of days			Germination in third test after storage for indicated number of days			Germination in fourth test after storage for indicated number of days			Germination in fifth test after storage for indicated number of days			Germination in sixth test after storage for indicated number of days		
	3	5	7	3	5	7	3	5	7	3	5	7	3	5	7	3	5	7
0° C																		
Minturki 1507...	6	26	28	0	0	1	0	7	11	12	32	37	36	64	83	75	79	85
Minhardi 1505...	1	9	14	0	0	2	0	0	0	0	0	2	1	7	21	10	21	32
Minard 2199...	10	34	44	3	6	8	3	9	13	2	9	11	1	6	14	28	41	53
Minhardi X Minturki 2312...	2	11	14	0	0	3	1	7	9	1	6	11	7	17	31	26	48	61
10° C																		
Minturki 1507...	4	15	20	0	0	3	0	0	4	9	17	29	22	40	58	85	93	94
Minhardi 1505...	0	4	10	0	0	0	0	0	2	3	4	12	1	3	7	13	35	41
Minard 2199...	9	29	49	1	2	3	1	9	15	2	7	14	2	22	24	21	34	35
Minhardi X Minturki 2312...	0	5	13	0	0	0	0	1	5	1	6	20	0	8	13	37	61	67
20° C																		
Minturki 1507...	3	16	20	1	3	5	8	22	33	43	59	72	76	88	90	94	100	---
Minhardi 1505...	3	11	14	0	0	2	0	2	1	4	10	2	6	20	33	61	67	---
Minard 2199...	13	35	48	0	0	1	0	5	8	15	52	62	24	63	78	89	98	98
Minhardi X Minturki 2312...	2	9	11	0	0	0	3	4	8	6	21	31	30	69	87	89	92	96
30° C																		
Minturki 1507...	4	17	26	68	80	91	82	95	97	97	99	99	99	100	---	100	---	---
Minhardi 1505...	4	13	21	0	5	12	8	45	59	86	96	99	95	100	---	98	100	---
Minard 2199...	6	26	31	65	73	82	73	91	94	98	100	---	100	---	---	100	---	---
Minhardi X Minturki 2312...	3	12	13	35	49	64	73	87	91	97	100	---	100	---	---	100	---	---
35° C																		
Minturki 1507...	7	19	23	45	57	70	84	97	98	97	100	---	99	100	---	100	---	---
Minhardi 1505...	0	4	5	0	5	9	5	53	68	92	99	99	98	100	---	97	97	97
Minard 2199...	8	27	40	23	30	39	56	83	86	85	97	98	98	99	100	100	---	---
Minhardi X Minturki 2312...	3	12	14	44	54	66	71	89	92	96	98	99	99	99	99	100	---	---

RIPE STAGE, COLLECTED JULY 16<sup>2</sup>

Storage temperature and variety	Germination in first test after storage for indicated number of days			Germination in second test after storage for indicated number of days			Germination in third test after storage for indicated number of days			Germination in fourth test after storage for indicated number of days			Germination in fifth test after storage for indicated number of days			Germination in sixth test after storage for indicated number of days		
	3	5	7	3	5	7	3	5	7	3	5	7	3	5	7	3	5	7
0° C																		
Minturki 1507...	---	---	---	26	37	67	60	82	86	94	98	99	96	100	---	100	---	---
Minhardi 1505...	---	---	---	0	0	2	2	3	8	6	24	34	2	14	39	33	59	60
Minard 2199...	---	---	---	52	58	74	44	57	63	76	87	91	70	93	96	89	93	96
Minhardi X Minturki 2312...	---	---	---	7	11	22	11	20	24	30	55	62	34	84	91	86	91	93
10° C																		
Minturki 1507...	---	---	---	33	46	78	78	83	87	95	100	---	95	99	99	99	100	---
Minhardi 1505...	---	---	---	0	1	13	0	0	2	6	14	22	1	15	33	47	56	58
Minard 2199...	---	---	---	22	30	52	27	48	58	83	88	93	86	96	99	89	95	96
Minhardi X Minturki 2312...	---	---	---	1	6	16	11	19	23	70	77	84	71	89	90	88	92	93
20° C																		
Minturki 1507...	10	41	46	78	94	97	100	---	---	99	99	99	99	100	---	100	---	---
Minhardi 1505...	0	3	15	4	13	25	4	12	13	33	69	74	55	91	94	90	93	95
Minard 2199...	10	40	45	52	59	73	78	86	91	97	99	100	100	---	---	98	99	99
Minhardi X Minturki 2312...	0	0	0	20	33	53	26	43	54	80	89	92	87	99	99	97	97	97
30° C																		
Minturki 1507...	---	---	---	96	98	99	100	---	---	100	---	---	100	---	---	100	---	---
Minhardi 1505...	---	---	---	3	11	26	62	78	82	100	---	---	100	---	---	100	---	---
Minard 2199...	---	---	---	87	90	95	100	---	---	100	---	---	100	---	---	100	---	---
Minhardi X Minturki 2312...	---	---	---	59	69	95	98	99	(?)	100	---	---	100	---	---	100	---	---
35° C																		
Minturki 1507...	---	---	---	96	98	99	100	---	---	100	---	---	100	---	---	100	---	---
Minhardi 1505...	---	---	---	3	14	40	83	89	92	100	---	---	100	---	---	100	---	---
Minard 2199...	---	---	---	91	91	95	100	---	---	100	---	---	100	---	---	100	---	---
Minhardi X Minturki 2312...	---	---	---	66	72	91	100	---	---	100	---	---	100	---	---	100	---	---

<sup>1</sup> The first test was made July 8, second test July 15, third test July 22, fourth test July 29, fifth test Aug. 5, and sixth test Aug. 12<sup>2</sup> The first test was made July 16, second test July 23, third test July 30, fourth test Aug. 6, fifth test Aug. 13, and sixth test Aug. 20.

TABLE 14.—*Germination percentages of spring wheat varieties harvested at different dates and stored at different temperatures, 1931*

[100 seeds taken for each test]

HARD-DOUGH STAGE, COLLECTED JULY 13<sup>1</sup>

Storage temperature and variety	Germination in first test after storage for indicated number of days			Germination in second test after storage for indicated number of days			Germination in third test after storage for indicated number of days			Germination in fourth test after storage for indicated number of days			Germination in fifth test after storage for indicated number of days			Germination in sixth test after storage for indicated number of days		
	3	5	7	3	5	7	3	5	7	3	5	7	3	5	7	3	5	7
0° C.:																		
Marquis 1239.....	---	---	---	0	0	0	0	0	0	0	2	3	0	0	3	0	3	11
Reward 2204.....	---	---	---	0	0	0	0	0	0	0	0	1	0	0	1	3	4	5
Kubanka 2310.....	---	---	---	19	33	36	0	22	44	0	10	42	1	18	45	0	1	44
Double Cross 2304.....	---	---	---	1	4	6	0	2	6	0	0	2	1	1	2	1	1	4
10° C.:																		
Marquis 1239.....	---	---	---	0	0	1	0	1	3	0	0	1	1	1	3	4	5	12
Reward 2204.....	---	---	---	0	1	2	1	1	3	0	1	2	1	4	6	0	2	7
Kubanka 2310.....	---	---	---	0	15	15	0	9	35	0	6	52	0	7	44	0	5	34
Double Cross 2304.....	---	---	---	0	6	7	0	3	7	0	1	4	0	0	2	0	0	13
20° C.:																		
Marquis 1239.....	0	2	8	0	0	2	0	0	2	0	2	4	16	35	28	42	58	
Reward 2204.....	0	0	0	3	3	3	0	0	1	0	1	3	11	41	62	53	73	89
Kubanka 2310.....	0	15	42	0	2	5	0	2	20	0	2	30	1	2	24	10	11	30
Double Cross 2304.....	0	4	16	1	1	1	0	0	0	0	0	2	2	2	0	1	2	
30° C.:																		
Marquis 1239.....	---	---	---	0	0	2	0	4	10	8	32	57	51	90	92	74	91	95
Reward 2204.....	---	---	---	4	8	11	21	51	58	67	90	98	95	97	97	96	98	100
Kubanka 2310.....	---	---	---	0	9	35	1	53	83	0	78	96	16	100	---	90	99	99
Double Cross 2304.....	---	---	---	0	0	1	0	9	19	4	13	36	11	91	95	92	100	---
35° C.:																		
Marquis 1239.....	---	---	---	0	2	3	2	13	26	32	55	69	46	84	94	74	83	96
Reward 2204.....	---	---	---	1	2	5	35	72	78	79	88	95	97	98	95	95	96	98
Kubanka 2310.....	---	---	---	0	23	65	0	58	92	2	91	100	15	100	---	67	100	---
Double Cross 2304.....	---	---	---	0	0	1	1	8	17	1	19	62	38	100	---	100	---	---

RIPE STAGE, COLLECTED JULY 22<sup>2</sup>

Storage temperature and variety	Germination in first test after storage for indicated number of days			Germination in second test after storage for indicated number of days			Germination in third test after storage for indicated number of days			Germination in fourth test after storage for indicated number of days			Germination in fifth test after storage for indicated number of days			Germination in sixth test after storage for indicated number of days		
	3	5	7	3	5	7	3	5	7	3	5	7	3	5	7	3	5	7
0° C.:																		
Marquis 1239.....	---	---	---	0	0	0	0	3	7	0	6	12	5	12	17	3	13	26
Reward 2204.....	---	---	---	2	2	8	0	4	16	15	36	43	24	44	53	42	57	68
Kubanka 2310.....	---	---	---	0	14	35	0	9	75	0	11	56	1	18	61	0	12	81
Double Cross 2304.....	---	---	---	0	0	2	0	0	2	0	0	1	0	0	0	0	0	4
10° C.:																		
Marquis 1239.....	---	---	---	0	0	1	0	0	3	2	9	10	4	12	17	12	23	38
Reward 2204.....	---	---	---	1	4	12	1	14	24	23	39	44	26	43	50	53	61	79
Kubanka 2310.....	---	---	---	0	6	17	0	3	50	0	4	27	0	8	42	0	5	84
Double Cross 2304.....	---	---	---	0	0	0	0	0	0	0	1	2	0	2	3	0	1	13
20° C.:																		
Marquis 1239.....	0	0	0	0	2	5	0	2	8	10	22	23	35	50	54	55	64	81
Reward 2204.....	0	3	4	4	25	27	20	41	54	62	77	84	78	84	86	82	87	92
Kubanka 2310.....	0	6	20	0	18	40	0	1	34	0	12	61	0	12	43	4	16	74
Double Cross 2304.....	0	0	0	0	0	0	0	0	3	0	1	1	1	3	4	1	9	40
30° C.:																		
Marquis 1239.....	---	---	---	10	22	23	8	22	42	55	95	95	86	100	---	100	---	---
Reward 2204.....	---	---	---	31	59	63	66	83	96	88	98	98	98	99	99	100	---	---
Kubanka 2310.....	---	---	---	0	39	56	4	58	97	23	99	100	79	97	98	89	98	---
Double Cross 2304.....	---	---	---	0	2	2	4	6	9	14	85	92	93	98	98	100	---	---
35° C.:																		
Marquis 1239.....	---	---	---	4	15	26	19	43	63	47	94	96	89	100	---	99	99	99
Reward 2204.....	---	---	---	24	47	53	59	82	90	82	99	99	94	100	---	100	---	---
Kubanka 2310.....	---	---	---	0	44	73	0	68	98	78	99	99	90	99	100	97	100	---
Double Cross 2304.....	---	---	---	0	3	3	1	6	30	59	96	96	99	100	---	100	---	---

<sup>1</sup> The first test was made July 13, second test July 20, third test July 28, fourth test Aug. 3, fifth test Aug. 10, and sixth test Aug. 17.<sup>2</sup> The first test was made July 22, second test July 30, third test Aug. 5, fourth test Aug. 12, fifth test Aug. 19, and sixth test Aug. 26.



# THE ESTIMATION OF THE HEAT PRODUCTION OF CATTLE FROM THE INSENSIBLE LOSS IN BODY WEIGHT<sup>1</sup>

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## INTRODUCTION

The mere instrumental errors incurred in measuring the heat elimination or the respiratory exchange of farm animals, amounting to 1 or 2 percent or less, are not factors of great moment among the many involved in the errors attached to the final significant calculations. The interpretive errors incurred in estimating heat production from heat elimination or respiratory exchange are possibly greater, although their precise magnitude is impossible to assess in the absence of a direct method of measuring heat production. However, what might be called the biological errors in animal calorimetry are by far the most serious, both because of their magnitude and because of the difficulty involved in their removal. These errors are incurred when successive comparative observations are made upon the same animal, but are probably revealed in their greatest magnitude only when similar observations are made upon a number of animals.

If a steer is put into a respiration chamber for 2 or 3 consecutive days under the same conditions of feeding and management, the daily estimates of heat production will of course not be identical, even when corrections for variable muscular activity, insofar as this is measurable, are applied. The average differences between the results for successive days will be 2 or 3 percent, while in individual cases deviations as high as 5 percent are not uncommon. Throughout the publications of the Institute of Animal Nutrition of Pennsylvania State College individual differences between animals with reference to activity, digestive ability, basal metabolism, and heat increment following feeding are repeatedly cited and discussed. From four recent publications (8, 9, 10, 11)<sup>3</sup> the writers have computed an average percentage difference of 7.1 between pairs of determinations on different steers of the net energy value of rations for maintenance, with individual differences as high as 13.9 percent. The earlier work of Armsby (1) shows even wider individual differences (up to 24 percent) and other laboratories have reported the same experience. Thus Kleiber (16) has calculated a coefficient of variation of 11 percent exhibited by a series of 11 determinations of the net energy value of starch reported by Kellner and Kühn. This coefficient means that the occurrence of deviations of as much as 22 percent between individual and average net energy values is not highly improbable.

<sup>1</sup> Received for publication Nov. 14, 1935, issued July 1936.

<sup>2</sup> The writers take great pleasure in acknowledging the assistance of members of the Animal Nutrition Division in carrying out the exacting experiments reported herein. Particular credit is due W. T. Haines, who assumed much of the responsibility of the care for the animals, the proper condition of the respiration apparatus, and the analyses of the chamber air.

<sup>3</sup> Reference is made by number (italic) to Literature Cited, p. 853.

Thus it appears that the significant technical problems of animal calorimetry relate, not to the diminution of instrumental errors, but more particularly to the diminution of the inherent biological errors or to the reduction of their effect upon the final calculations. The latter plan is the more practicable and the one more likely to justify itself. The effect of the biological errors upon the final average results of calorimetric work (net energy values, maintenance, and production requirements) may be diminished by increasing the number of animals upon which measurements are made. But, for purely practical reasons, any considerable increase in the number of experimental subjects must be accompanied by a decrease in the time, labor, and expense involved in securing individual measurements.

What appears to be a promising method of estimating the heat production of animals in a simple manner rests upon the determination of the so-called "insensible perspiration" or, better, "insensible loss in body weight." This loss in weight is the result of the gaseous exchange between an animal and its environment, and is equal to the difference in weight between the gases emitted from the body (carbon dioxide, water vapor, and methane) and the oxygen taken in. Over short periods the insensible loss in weight may be measured by placing the animal upon a sensitive balance and noting the loss in weight, but this simple method is inapplicable over long periods, since normally sensible accretions (food and water) and excretions (feces and urine) to and from the body will occur. The insensible loss in weight over periods of days or weeks may be obtained by weighing the animal at the beginning and at the end of the desired period, together with all food and water consumed and urine and feces excreted. The insensible loss in weight is then computed from the following equation:

$$I = (B_1 + N + W) - (B_2 + U + F)$$

in which  $I$  is the insensible loss in body weight,  $B_1$  and  $B_2$  the initial and final body weights, respectively,  $N$  the food intake,  $W$  the intake of water,  $U$  the output of urine, and  $F$  the fecal excretion. If the insensible loss in body weight may be used successfully in the prediction of heat production, it is evident that a simple gravimetric method has replaced the elaborate and laborious technique of direct and indirect calorimetry.

The relation between the insensible loss in weight and the heat elimination of an animal relates entirely to one factor in this loss, namely, the water vapor. The significance of this factor in the prediction of the total heat elimination resides in the observation, repeatedly recorded in the literature, that the percentage of heat eliminated from an animal in the vaporization of water from the skin and the respiratory tract is remarkably constant, biologically speaking, under more or less restricted conditions. A historical discussion of this literature has been prepared by Benedict and Root (4), while discussions of the nature of the insensible loss in weight and of the conditions affecting it have been offered by Benedict and Wardlaw (5), Benedict (2), and Kuno (19).

The relation between the water vaporized from the body and the insensible weight loss is a function of the respiratory quotient and of the percentage of the heat elimination represented in the vaporized water, disregarding for the moment the excretion of methane in ruminants.

It may be shown <sup>4</sup> that the ratio of vaporized water to insensible loss is represented by the following expression:

$$\frac{W}{I} = \frac{ce}{ce + 1.1466r - 0.8288}$$

<sup>4</sup> The derivation of formulas concerned with the relations existing between the insensible loss in body weight, the water vaporized, and the heat production follows

1 The ratio of water vaporized to the insensible loss in body weight

Let  $a$  = liters of  $\text{CO}_2$  produced

$b$  = liters of  $\text{O}_2$  consumed

$c$  = fraction of total heat lost as vaporized water

$d$  = latent heat of vaporization per gram of water

$e$  = heat equivalent of a liter of oxygen consumed

$$= 4\,686 + 1\,23 \left( \frac{a}{b} - 0.707 \right)$$

$h$  = heat produced =  $be$

$w$  = weight of water vaporized

$i$  = insensible loss in body weight

Then, total heat of vaporization =  $bce$

total water vaporized =  $w = \frac{bce}{d}$

$$\text{insensible loss} = i = \frac{bce}{d} + 1\,9769a - 1\,4290b \quad (1)$$

$$\frac{w}{i} = \frac{\frac{bce}{d}}{\frac{bce}{d} + 1.9769a - 1.4290b} \quad (2)$$

$$= \frac{ce}{ce + 1.9769 \frac{a}{b} \cdot d - 1.4290d} \quad (3)$$

If  $d = 0.580$

$$\text{Then } \frac{w}{i} = \frac{ce}{ce + 1.1466 \frac{a}{b} - 0.8288} \quad (4)$$

2 The fraction ( $c$ ) of total heat lost as vaporized water

From equation (4)

$$ice = wce + 1.1466 \frac{a}{b} \cdot w - 0.8288w \quad (5)$$

$$wce - ice = 0.8288w - 1.1466 \frac{a}{b} \cdot w \quad (6)$$

$$c = \frac{0.8288w - 1.1466 \frac{a}{b} \cdot w}{we - ie} \quad (7)$$

3 The water vaporized

$$c = \frac{0.580w}{h} \quad (8)$$

Combining (5) and (8)

$$\frac{0.580w^2e}{h} + 1.1466 \frac{a}{b} \cdot w - 0.8288w = \frac{0.580iew}{h} \quad (9)$$

Dividing by  $w$  and multiplying by  $h$

$$0.580we + 1.1466 \frac{a}{b} \cdot h - 0.8288h = 0.580ie \quad (10)$$

Dividing by 0.580 and transposing

$$we = 1.4290h - 1.9769 \frac{a}{b} \cdot h + ie \quad (11)$$

$$\frac{1.4290h - 1.9769 \frac{a}{b} \cdot h + ie}{w} = e \quad (12)$$

4 The heat produced

From (8)

$$w = \frac{ch}{0.580} \quad (13)$$

Combining (12) and (13)

$$\frac{che}{0.580} = 1.4290h - 1.9769 \frac{ah}{b} + ie \quad (14)$$

Simplifying

$$h = \frac{ie}{1.7241ce + 1.9769 \frac{a}{b} - 1.4290} \quad (15)$$

NOTE.—The absolute density of  $\text{CO}_2$  is equivalent to 1.9769 g. per liter according to the International Critical Tables (30, v 3). If, as Kleiber (18, p. 11) believes, the theoretical value of 1.9652 is preferable to use in this connection, because of the small concentration in which  $\text{CO}_2$  occurs in chamber air, the formulas developed above may be readily changed accordingly.

in which  $c$  is the fraction of the total heat that is lost as vaporized water,  $e$  is the calorie equivalent of a liter of oxygen consumed (and is a function of  $r$  for respiratory quotients between 0.7 and 1), and  $r$  is the respiratory quotient. For purposes of illustration, the value of  $\frac{W}{T}$ , expressed as a percentage, is given in table 1 for various values of  $c$  and  $r$ .

TABLE 1.—*The percentage of the insensible weight loss represented by vaporized water for various values of  $c$  and  $r$*

Percentage of heat lost as vaporized water (c×100)	Percentage loss at respiratory quotient (r) of—						
	0.707	0.75	0.80	0.85	0.90	0.95	1.00
20.....	102.0	96.8	91.6	87.0	82.9	79.3	76.1
25.....	101.6	97.4	93.1	89.3	85.8	82.7	79.9
30.....	101.3	97.9	94.2	90.9	87.9	85.2	82.7
35.....	101.1	98.2	95.0	92.1	89.5	87.0	84.8
40.....	101.0	98.4	95.6	93.0	90.7	88.4	86.4
45.....	100.9	98.6	96.1	93.8	91.6	89.6	87.7
50.....	100.8	98.7	96.4	94.3	92.4	90.5	88.8

For a respiratory quotient of 0.723, the weight of  $\text{CO}_2$  emitted and of  $\text{O}_2$  consumed are equal, so that at this respiratory quotient the insensible loss measures exactly the vaporized water. At lesser respiratory quotients the insensible loss is less than the weight of vaporized water, while at greater respiratory quotients it is greater, until for a respiratory quotient of 1, the vaporized water accounts for only 76 to 89 percent of the insensible loss as the percentage of heat lost in vaporization varies from 20 to 50. It is evident also that as the latter percentage increases, the weight of vaporized water tends to approach the insensible loss in weight at any respiratory quotient.

Although Benedict and Root (4) proposed a method of estimating the basal heat production of adult humans from the insensible loss in body weight, and Levine and others (22, 23, 24, 25) proposed a similar method for infants, Newburgh and his associates (14, 32, 33) have perfected the method and extended it to the determination of the total heat production for the day and for even longer periods, and have studied the effect of many conditions, in both nude and clothed subjects, on the relationship of insensible water and heat eliminated. Their success in particular inspired the investigations reported in this paper.

Previous studies of the relationship between the insensible loss in body weight and the heat production of cattle have not indicated a correlation sufficiently close to insure an acceptable prediction of the latter from the former. However, both Benedict and Ritzman (3, p. 75) and Kriss (17, 18) expressed the opinion that a variable environmental temperature may have been a disturbing factor, while Kriss further noted the disturbing effect of a variable hair coat, an observation confirming previous work from the same laboratory (7) as well as the results of Lefèvre and Auguet (21) on sheep. In the nude human subject Wiley and Newburgh (33) noted a steadily increasing percentage of heat lost in vaporization as the environmental temperature increased up to  $30^\circ$  to  $31^\circ \text{C}$ ., at which point visible perspiration appeared and initiated a marked acceleration in the rate of increase.

In the clothed subject, however, the percentage was not appreciably disturbed within the range of 18° to 30° C. Kayser (15) observed in pigeons and rabbits a curvilinear relationship between environmental temperature and the ratio of vaporized water to oxygen consumed, while Lefèvre and Auguet (21) showed that in sheep also, whether sheared or unsheared, the percentage of heat lost as latent heat of vaporization increased with the surrounding temperature.

While increasing environmental humidity continuously depresses the evaporation of water from the skin of the nude human subject (33), in the clothed human subject a variation in humidity from 20 to 60 percent has no such effect. Also, in ewes carrying a heavy fleece, the evaporation of water is not depressed by increasing the humidity of the surrounding air until values of 80 to 90 percent are attained, according to Lefèvre and Auguet (20). On the other hand, extreme dehydration of the body may depress the evaporation of water from the surface (12, 26, 28, 31). The effect of the intensity of heat production on the loss of heat in the vaporization of water does not seem to be considerable, if appreciable at all (13, 33). This phase of the subject, however, evidently requires further study.

Undoubtedly these and other disturbing factors are responsible for the occasional erratic values obtained for the ratio of basal insensible perspiration to basal metabolism in humans observed by Magendantz (27), as well as for the many other instances of the same nature cited by him from other laboratories.

#### PLAN OF THE EXPERIMENT

The accuracy of any prediction of the heat production of an animal from its insensible loss in body weight rests upon the constancy of the proportion of the total heat emitted as vaporized water. It seems evident from previous work that this proportion varies with environmental conditions, particularly with environmental temperature, and that animals other than the clothed human subject are particularly subject to these environmental changes.

The steers serving as subjects of these experiments were, therefore, confined in an air-conditioned and insulated room, in which temperature could be maintained constant within 2° or 3° F. and relative humidity within 5 or 6 percent. The urine and feces were collected beneath the floor of the room in such a way as to minimize the evaporation of water. The steers were weighed daily on scales accurate to 0.25 pound, situated immediately outside of the stall room door. The day's urine and feces were weighed, for each steer, at the exact time the body weight was taken. The time spent by each steer in the standing position was measured by an electric clock, the current to which was shut off by an arrangement of chains and pulleys as long as the steer remained in the recumbent position.

Water was offered the steers daily after securing their body weights, and the amount consumed was determined by immediately reweighing. The steers were fed twice daily. In most of the experiments reported below, both feed and water were brought to the temperature of the air-conditioned room before they were consumed.

The ration fed, which was varied in different experimental periods, consisted of varying proportions of corn and chopped alfalfa hay with small additions of salt (28 or 56 g daily). In periods in which small

proportions of alfalfa were fed, small amounts of bonemeal and cod-liver oil were also given, while in the later periods, when it was desired to increase feed consumption to the limit of capacity, brown sugar was fed at the rate of 100 g per meal. However, this attempt to increase the palatability of the ration was not accompanied by any appreciable measure of success.

The plan of the experiment was to determine the insensible loss in body weight of each steer in a period on constant feed, lasting 14 days or longer, and to measure also the heat production and gaseous exchange of the steer on the same level of feed, generally during a 72-hour confinement in the respiration chamber previously described (29). The latter determination was made at a time (either before or after) as close to the former as was feasible with the equipment available. During the former period (or for at least 2 weeks of it) the urine and feces of the steer were quantitatively collected, and samples of them, as well as of the feed, were analyzed for nitrogen and their heats of combustion were determined in the bomb calorimeter. Two such comparative determinations between insensible loss in body weight and heat production were made upon three of the four steers, and three determinations upon the other.

In the preliminary experiments, involving maintenance levels of feeding, the methane production was not determined and no account of it was taken in the computations of heat production or in the interpretation of the insensible loss in weight. However, in the main experiments the methane content of the outgoing air was determined gasometrically, and the methane production was considered in all the computations of indirect calorimetry, as previously described (29), and in the interpretation of the insensible loss in body weight.

#### RESULTS OF THE EXPERIMENT

In preliminary experiments, extending over 2 years, an investigation was made of the factors affecting the insensible loss in body weight, since these would be the factors that must be controlled in all later work. The first factor investigated was the environmental temperature. The results are briefly summarized in table 2. The heat-production values in column 12 of the table are derived from an average for each steer of either three or four respiration tests, each of which extended over 48 hours. These tests were all made at chamber temperatures of 75° F. or above. Small corrections were applied to these averages for differences in body weight and times spent in the standing position for the several periods. The average heat productions, expressed to a standard day of 12 hours standing were: Steer 1, 9,744 calories for a body weight of 474 kg; steer 2, 9,943 calories for a weight of 480 kg; steer 3, 7,871 calories for a weight of 331 kg; steer 4, 7,936 calories for a weight of 346 kg. The corrections applied to these figures for body weight were made on the basis of a basal metabolic rate (the steer being in the lying position) of 1,581 calories per square meter of body surface, the surface area being computed by Brody's formula (6) for beef cattle,  $S_m^2 = 0.13 W^{0.55}$  kg. The corrections for position (standing and lying) were made on the basis of an increment of 62 calories per 500 kg per hour for the standing position (29). Throughout these tests the steers were subsisting on an approximate maintenance ration consisting of equal parts of corn and alfalfa hay.

The weight of water vaporized was computed from equation (12) of footnote 4, p. 839. The respiratory quotients were very close to 1 in all cases, being 1.000 for steer 1, 0.988 for steer 2, and 0.995 for steers 3 and 4.

TABLE 2.—*The effect of room temperature on the insensible loss in weight and the vaporization of water from the body*

Steer no	Room temperature	Humidity	Average body weight	Feed eaten daily	Water drunk daily	Daily urine	Daily feces	Daily insensible loss in weight	Water vaporized		Heat produced daily	Heat of vaporization	
									Per day	As ratio of insensible loss		Total	As ratio of total heat
	° F	Percent	Kilo-grams	Kilo-grams	Kilo-grams	Kilo-grams	Kilo-grams	Kilo-grams	Kilo-grams	Percent	Calo-ries	Calo-ries	Percent
1	69	51	469	4	16.07	8 10	3 75	8 20	7.119	86 8	9,954	4,129	41 5
	58	64	481	4	12.16	7 17	3.51	4.97	3.900	78 5	9,862	2,262	22.9
	52	76	473	4	11.43	6.70	3.64	4.45	3.384	70.0	9,815	1,963	20.0
	43	88	474	4	14.82	8 67	4.13	4.61	3.575	77 5	9,538	2,073	21.7
2	69	51	480	4	10.30	2 35	3.54	7 57	6.559	86 6	9,701	3,804	39.2
	58	64	488	4	5 81	2 26	3.22	4 54	3.522	77.6	9,776	2,043	20.9
	52	76	479	4	5 37	2 27	3.26	3 93	3.918	74 2	9,713	1,692	17.4
	43	88	474	4	7.92	3 61	3 69	3 49	2.480	71 1	9,696	1,438	14.8
3	69	51	332	3	9 97	3 78	2.55	6 03	5 191	86 1	7,855	3,011	38.3
	58	64	340	3	8 42	5 46	2 39	4.00	3.154	78 4	7,928	1,829	23.1
	52	76	330	3	7 35	3 51	2 55	3.61	2 770	76.7	7,865	1,607	20.4
	43	88	322	3	10.39	6 64	2 83	3.00	2 107	72 2	7,802	1,257	16.1
4	69	51	342	3	9.04	1 96	2 12	6.59	5 760	87.4	7,778	3,341	42.9
	58	64	355	3	8 15	3 81	2 56	4.14	3 268	79.7	7,835	1,913	24.3
	52	76	348	3	5 82	2 36	2 72	3.69	2 838	76 9	7,838	1,646	21.0
	43	88	340	3	6 95	2 68	3.21	3.38	2 553	75 5	7,747	1,481	19.1

It will be noted that the insensible losses in body weight (table 2, column 9), the vaporized water, both the absolute values and the percentages on the insensible losses in weight, and the percentages of heat lost in the vaporization of water, decreased with one exception (steer 1, from periods 3 to 4) as the room temperature decreased. The averages of the latter percentages for all four steers were, in the order of decreasing temperatures, 40.5, 22.8, 19.7, and 17.9. It is quite possible that at the lowest temperature, 43° F., the steers were below their critical temperatures, in which case the calculated percentages would be too low, since the heat productions would be higher than those assumed on the basis of determinations made at higher temperatures in the respiration chamber.

In the experiments just reviewed, no particular attention was paid to the control of the humidity of the room air, the humidities actually obtained rising with decreasing temperatures because of the increasing difficulty of dehumidifying the air. With the temperature maintained at 68° to 70° F., tests were made of the effect upon the insensible loss in body weight of varying the humidity of the air. The same steers were used and they were maintained upon the same rations. The comparisons given in table 3 relate to short periods immediately preceding and immediately following a marked change in relative humidity.

Unfortunately the results of this test are indecisive. A comparison of periods 1 and 2, differing the most in relative humidity, would support the conclusion that a drop from 80 to 47 percent exerted no

effect on the vaporization of water from the bodies of the steers, three of the steers, in fact, showing a decrease in insensible loss rather than the expected increase if any effect at all were produced. On the other hand, in periods 3 and 4, an increase in relative humidity from 59 to 78 percent was associated in all steers with a more or less considerable drop in the insensible loss in body weight, and presumably in the vaporization of water. It is perhaps fair to conclude that if the vaporization of water from the bodies of these steers is inversely correlated with relative humidity between the limits of 47 and 80 percent, the effect is so slight that it may be obscured (or greatly accentuated) by uncontrolled factors.

TABLE 3—*The effect of relative humidity on the insensible loss in body weight of steers at chamber temperatures of 68° to 70° F.*

Steer no.	Period		Relative humidity	Insensible loss in weight	Steer no.	Period		Relative humidity	Insensible loss in weight
	No	Days	Percent	Kilo-grams		No.	Days	Percent	Kilo-grams
1.-----	1	13	80	6.40	3.-----	1	10	80	3.74
	2	8	47	6.65		2	4	47	3.25
	3	6	59	5.59		3	9	59	2.97
	4	8	78	5.39		4	10	78	2.80
2.-----	1	5	80	6.04	4.-----	1	6	80	4.62
	2	9	47	5.73		2	10	47	4.51
	3	7	59	4.81		3	7	59	3.75
	4	10	78	4.47		4	8	78	3.14

As these preliminary tests progressed it was evident that another factor (or other factors) than the temperature and humidity of the surrounding air was modifying the vaporization of water from the steers and hence the insensible loss in body weight. This uncontrolled factor (or factors) was evident only over a considerable period, exerting a progressive effect, but not disturbing the day-to-day variation. The data of table 3 reveal the type of effect under discussion: In periods 3 and 4, the insensible losses of all steers were lower than in periods 1 and 2, although the ration was the same and the body weights were nearly so.

The observations of the Institute of Animal Nutrition at Pennsylvania State College (7, 17, 18) on the effect of shearing on the insensible loss in body weight of steers, as well as those of Lefèvre and Auguet (21) on the effect of shearing sheep on the vaporization of water, suggested a solution of this problem. Accordingly, the effect of shearing on four young steer calves was studied, with the results summarized in table 4. The experimental periods immediately preceded or followed shearing and were 14 days in length except the final period (after shearing), which was only 8 days in length. In comparable periods, all conditions were kept the same except the hair coat of the steers.



TABLE 4.—*The effect of shearing on the daily insensible loss in body weight of steers and on certain features of their behavior, when fed 2 different rations*

DAILY RATION. 24 KG OF CORN PLUS 3.4 KG OF CUT ALFALFA HAY

Steer no	Condition	Average body weight	Daily gain in weight	Water consumed daily	Time spent standing per day	Pulse rate	Daily insensible loss in body weight
		Kilo-grams	Kilo-grams	Kilo-grams	Hours		Kilo-grams
1-----	{ Before shearing.....	258	+0 34	10 36	9 32	64.6	6 59
	{ After shearing.....	270	+ 94	9 30	8 91	60 3	5 08
2-----	{ Before shearing.....	257	+ 66	10 30	10 09	67 3	5 50
	{ After shearing.....	268	+ 55	9 67	11 71	60.1	5 21
3-----	{ Before shearing.....	243	+ 21	10 71	5 26	50.3	5 60
	{ After shearing.....	254	+1 04	9 81	5 61	52.2	4 56
4-----	{ Before shearing.....						
	{ After shearing.....	238	+ 73	10 57	6 94	59.6	5 52

DAILY RATION. 36 KG OF CORN PLUS 12 KG OF CUT ALFALFA HAY

1-----	{ Before shearing.....	317	+ 65	9 58	11 77	66.7	7 36
	{ After shearing.....	327	+ 71	7 45	8 10	61 9	5 29
2-----	{ Before shearing.....	300	+ 26	9 40	11 15	64.4	6 83
	{ After shearing.....	304	+ 93	8 58	9 75	60 5	5 27
3-----	{ Before shearing.....	297	+ 23	8 85	8 87	56 4	6 00
	{ After shearing.....	305	+ 37	7 90	7 10	54.3	5 24
4-----	{ Before shearing.....	289	+ 10	8 94	8 10	63.0	7 74
	{ After shearing.....	296	+ 91	7 40	7 46	60.5	5 44

It is evident that shearing decreased the insensible loss in body weight, the observed decreases ranging from 5.3 to 29.7 percent and averaging 19.8 percent. This decrease may have been the result of two factors (1) a decrease in skin temperature following removal of the insulating hair, (2) a decrease in restlessness of the animal due to a removal of scurf and dirt from the skin. The advantage of periodically removing scurf and dirt from the skin of steers on a long-time experiment is obvious, but this factor was not an important one in this case, since in another test currying alone failed to exert an appreciable effect on the insensible loss in body weight. Hence, the decrease in skin temperature must have been the effective factor in shearing.

The decrease in skin temperature occasioned a decreased loss of water by evaporation from the skin for each calorie of heat dissipated. As a result, less water was consumed daily in all cases after shearing than before; but this reduction in water intake was generally less than the reduction in water vaporized from the body. The daily volume of urine excreted (not shown in the table) was also generally less after shearing than before; hence shearing must have induced a hydration of the bodies of the steers, an effect that extended over many days. This storage of water probably accounts for the greater gain in body weight after shearing than before in all cases but one (the steer showing the least effect of shearing), and this greater gain after shearing would continue until the appetite for water adjusts itself to the decreased demand.

The advantages of frequent shearing in determining insensible losses in body weight representative of a given steer, ration, and set of environmental conditions are marked, and their presence is clearly evident from the data of table 4 in the much greater uniformity of

the insensible loss values obtained after shearing as compared with those obtained before. Thus, in the second comparison in the table the standard deviation of the insensible losses before shearing was 0.65 kg and the coefficient of variation 9.3. The same statistics after shearing were 0.076 kg and 1.4.

In securing constant and representative values for the insensible loss in body weight of steers, a fairly constant intake of water was found desirable. Abstention from water for several days will cause a dehydration of the body, irregular and abnormally low vaporization of water, and unrepresentative insensible losses in body weight. One steer had to be removed from the preliminary experiments because of a very irregular water appetite.

The principal experiments, concerned with the possibility of predicting the heat production of steers from their contemporaneous insensible loss in body weight, were performed in the following year upon the same four steers used in the last preliminary test (table 4). The tests were carried out with the observance of all the precautions indicated by the preliminary studies. The room temperature was kept constant at 76° F. and the relative humidity at 52 to 54 percent. The steers were sheared before each determination of the insensible loss in body weight. The control of air conditions in the respiration chamber was not feasible, but the temperature was allowed to vary only between 74° and 81° or 82°. The humidity, under no control whatever, averaged 78 to 80 percent. It is not believed that these chamber conditions either depressed or accelerated appreciably the metabolic rate obtaining during the determination of the corresponding insensible loss in body weight.

The rations used have already been described. Their content of metabolizable energy was determined in two tests upon each of the four steers, with the results given in table 5. The corn contained an average of 1.62 percent of nitrogen and 3.91 calories of gross energy per gram; the alfalfa hay, 2.45 percent of nitrogen and 4.04 calories of gross energy per gram; for the brown sugar, these values were 0.055 percent of nitrogen and 3.65 calories per gram. It is worthy of note that steers 1 and 4 were distinctly better utilizers of feed energy than were steers 2 and 3. Steer 3 was almost continuously bloated throughout these experiments.

TABLE 5.—The metabolizable energy content of the experimental rations

Steer no.	Daily ration			Gross energy in—				Metabolizable energy		
	Corn	Alfalfa hay	Sugar	Daily feed	Daily feces	Daily urine	Daily methane	Total per day	Per kilo-gram of feed	Per-centage of gross energy
	Kilo-grams	Kilo-grams	Kilo-grams	Calories	Calories	Calories	Calories	Calories	Calories	Calories
1	3.84	0.96	0.0	18,804	3,587	470	1,174	13,573	2,828	72.2
	4.56	2.28	.2	27,881	7,328	514	2,214	17,825	2,608	63.9
2	3.40	1.70	0	20,274	6,334	392	1,161	12,387	2,401	61.2
	3.92	1.95	.2	23,969	8,674	479	1,521	13,295	2,193	55.5
3	3.84	.96	0	18,804	5,252	376	1,321	11,875	2,474	63.2
	3.40	1.70	.2	20,887	7,236	462	1,901	11,228	2,202	53.8
4	3.84	.96	0	18,804	3,537	423	1,861	13,043	2,717	69.4
	3.40	1.70	.2	20,887	5,860	475	1,500	13,052	2,559	62.5

The insensible losses in body weight of the steers, together with the data upon which the computation of these losses is based, are summarized in table 6. The only feature of these results requiring comment here is the variability of the day-to-day insensible losses in these various experimental periods. The pertinent standard deviations and coefficients of variation are given in the last two columns of the table. The coefficients of variation range from 3.43 to 8.84, and average 6.20, a satisfactorily low figure. The day-to-day variations in these experiments may be compared with the only record of its kind found in the literature, a 38-day record of a human subject reported by Johnston and Newburgh (14). The subject, a normal man 32 years old, spent practically all of his time in bed, his activity being rigidly restricted. The temperature of the room was kept close to 72° F. during the day. Considering only the last 30 days during which the intake of food was constant, the average daily insensible loss was 976, the standard deviation was 69.5, and the coefficient of variation was 8.71. In later work, Newburgh, Wiley, and Lashmet (32) report daily estimations of the insensible (vaporized) water for seven subjects, pursuing their usual routine duties, but subsisting upon a constant diet approximating closely their maintenance requirements. Ruling out the values obtained on extra cold days and days on which unusual work was done, the coefficients of variation for 15 periods of 6 to 15 days each ranged from 3.21 to 13, and averaged 8.4.

TABLE 6.—*The insensible loss in body weight of the steers per day and data upon which computations were based*

Steer no.	Period no.	Length of period	Average body weight		Average daily gain in weight	Time in standing position	Feed consumed		Water drunk	Urine	Feces	Insensible loss in body weight	Variability of daily insensible losses	
			Kilo-grams	Kilo-grams			Kilo-grams	Kilo-grams					Standard deviation	Coefficient of variation
		Days				Hours								
1.....	1	22	414	+0.35	6 14	4 85	7 25	3.11	2.41	6.23	0.436	6 99		
	2	28	481	+ .71	6 57	7.11	13.51	5.23	5.45	9.23	.691	7 49		
	1	14	379	+ .29	10 67	5.13	9.10	3.89	4 18	5 87	.248	4 22		
2.....	2	21	396	+ .26	11 12	6 18	10 65	4 31	5.48	6 78	.297	4 38		
	1	24	377	+ .33	7 66	4.85	9.46	4.88	3.44	5.66	.436	7.70		
	2	21	421	+ .69	7 65	5 36	11.13	5.09	4.61	6.10	.639	8.84		
3.....	1	30	393	+ .25	7 03	4 85	8 32	3 33	2.56	7 03	.609	7 24		
	2	16	426	+ .68	7 34	5.33	12 55	5.17	4 27	7 76	.266	3.43		
	3	14	431	+ .21	7 27	5 36	11 72	5 49	4.08	7 30	.404	5 53		

The coefficients of variation given in table 6 are considerably lower and more uniform than those calculated from the results of the preliminary investigations. For example, in the tests summarized in table 2, the coefficients of variation of the daily insensible losses in weight ranged from 2.91 to 12.56, averaging 7.06.

The results of the respiration experiments on the four steers are summarized in table 7. The first three experiments lasted for 48 hours, but all others extended through 72 hours. The successive 24-hour values are all recorded in the table. The average heat productions are ordinarily the average of the corresponding 2- or 3-day

values, although in three instances, one of the three daily values was omitted from the average because it was considerably higher than the other two, probably because of exceptionally greater activity. In one of the cases (steer 2, experiment 118-35) objective evidence of exceptional activity was obtained.

TABLE 7.—*The respiratory exchange and heat production of steers per day and data upon which computations were based*<sup>1</sup>

Steer no	Experiment no	Average body weight	Day of test	Daily feed			Time in standing position	Oxygen consumed	Carbon dioxide produced	Methane produced	Respiratory quotient	Heat production
				Corn	Alfalfa	Sugar						
		Kilo-grams		Kilo-grams	Kilo-grams	Kilo-grams	Hours	Liters	Liters	Kilo-grams		Calories
1-----	116-35	430	First.....	3 84	0 96	0 0	7 75	1,809	1,814	0 087	1 003	9,051
			Second.....	3 84	.96	0	8 38	1,783	1,881	0 090	1.055	9,029
			Average..	3 84	.96	0	8 06	1,796	1,847	.088	1 028	9,040
			First.....	4 60	2 30	2	8 96	2,452	2,720	.169	1.109	12,538
	119-35	481	Second.....	4 60	2 30	2	7 70	2,438	2,676	.168	1 098	12,438
			Third.....	4 60	2 30	2	-----	2,461	2,666	.161	1 083	12,521
			Average..	4 60	2 30	2	8 33	2,450	2,687	.166	1.097	12,499
			First.....	3 40	1 70	0	8 04	1,705	1,818	.086	1.066	8,659
	118-35	366	Second.....	3 40	1 70	0	6 96	1,683	1,865	.088	1 108	8,625
			Third.....	3 40	1 70	0	(13 06)	(1,884)	(1,971)	(.093)	(1.046)	(9,527)
2-----			Average <sup>2</sup> .	3 40	1 70	0	6 50	1,694	1,842	.087	1.087	8,642
			First.....	4 00	2 00	.2	10 10	1,834	2,028	.097	1 106	9,394
			Second.....	4 00	2 00	.2	11 36	1,953	2,199	.126	1.126	10,032
			Third.....	4 00	2 00	.2	11 49	2,132	2,318	.120	1.087	10,871
	122-35	397	Average <sup>3</sup> .	4 00	2 00	.2	10 98	1,973	2,182	.114	1.106	10,099
			First.....	3 84	.96	0	9 23	1,866	1,927	.096	1 033	9,402
			Second.....	3 84	.96	0	10 42	2,015	2,037	.102	1.011	10,099
			Average..	3 84	.96	0	9.82	1,940	1,982	.099	1 022	9,750
	121-35	415	First.....	3 40	1 70	2	9.18	2,061	2,126	.146	1 032	10,344
			Second.....	3 40	1 70	.2	6 93	2,005	2,110	.134	1 052	10,122
Third.....			3 40	1 70	2	6.86	1,968	2,039	.162	1.036	9,868	
3-----			Average <sup>4</sup> .	3 40	1 70	.2	7 66	2,011	2,092	.147	1 040	10,111
			First.....	3 84	.96	0	9.73	2,040	2,228	.134	1.092	10,400
			Second.....	3 84	.96	0	8.84	1,996	2,262	.136	1 133	10,265
			Average..	3 84	.96	0	9.28	2,018	2,245	.135	1.112	10,332
	120-35	426	First.....	3 40	1 70	.2	6.55	2,132	2,316	.119	1 086	10,874
			Second.....	3 40	1 70	.2	12 76	2,175	2,302	.107	1 058	11,031
			Third.....	3 40	1 70	2	(10.96)	(2,636)	(2,597)	(.106)	(.985)	(13,152)
			Average..	3 40	1 70	.2	11.29	2,154	2,309	.113	1 072	10,952
	123-35	441	First.....	3 40	1 70	.2	8.88	2,054	2,162	.109	1 053	10,394
			Second.....	3 40	1 70	.2	9.33	2,070	2,244	.114	1 084	10,552
Third.....			3 40	1 70	.2	(9.63)	(2,287)	(2,356)	(.119)	(1 030)	(11,517)	
Average..			3 40	1 70	2	9 11	2,062	2,203	.112	1.068	10,473	

<sup>1</sup> The values enclosed in parentheses are not included in the respective averages

<sup>2</sup> There were Orts in this 3-day test amounting to 630 g., dry weight

<sup>3</sup> There were Orts in this 3-day test amounting to 360 g., dry weight.

<sup>4</sup> Orts weighing 70 g., dry weight.

In table 8, the data of tables 6 and 7 have been utilized in estimating the weight of water vaporized from the steers daily. In combining the two sets of data, the observed heat productions have been corrected to the average body weights and standing records of the periods serving for the determination of the insensible losses in weight, by a method described on page 842. These corrections were all relatively small, ranging from +7 to +324 calories daily. The

weights of respiratory gases (but not of methane) have been similarly corrected, increasing or decreasing the observed values in the same proportions that the corresponding observed heat productions were increased or decreased. While a theoretically better (but much more complicated) method might have been used in making these corrections, the end results would have been very nearly the same.

TABLE 8.—*Computation of percentage of heat lost as vaporized water, the results being expressed on the daily basis*

Steer no	Period no.	Insensible loss in body weight	Results of respiration experiment <sup>1</sup>					Vaporized water	Latent heat of vaporization	Heat lost as vaporized water
			Experiment no.	Carbon dioxide produced	Methane produced	Oxygen consumed	Heat produced			
		Kilo-grams		Kilo-grams	Kilo-grams	Kilo-grams	Calories	Kilo-grams	Calories	Percent
1	1	6 23	116-35	3.55	0.09	2 50	8,795	5 09	2,952	33.6
	2	9 23	119-35	5 27	.17	3 47	12,391	7 26	4,211	34.0
2	1	5.87	118-35	3.78	.09	2 51	8,966	4 51	2,616	29.2
	2	6.78	122-35	4 31	.11	2 82	10,106	5 18	3,004	29.7
3	1	5 66	115-35	3.85	.10	2 73	9,593	4 44	2,575	26.8
	2	6.10	121-35	4.16	.15	2 89	10,163	4 68	2,714	26.7
4	1	7 03	117-35	4 34	.13	2 82	10,093	5 38	3,120	30.9
	2	7 76	120-35	4 48	.11	3 02	10,736	6 19	3,590	33.4
	3	7 30	123-35	4 28	.11	2 89	10,281	5 80	3,364	32.7

<sup>1</sup> Except for methane, these values, as compared with those in table 6, are corrected for differences in body weight and in time spent in the standing position between the respiration tests and the periods of insensible loss determination (p. 842).

The vaporized water was computed by subtracting from the insensible loss in body weight the weights of carbon dioxide and of methane excreted, and adding to the remainder the weight of oxygen absorbed. Multiplying the weight of vaporized water by 0.58 gives the heat of vaporization. Expressing the heat of vaporization in each case as a percentage of the total heat produced (and within a small error the total heat eliminated) results in the values recorded in the last column of table 8. These percentages are the most significant results of the experiment.

The percentages of heat output represented by the vaporized water are not constant among the different steers, but for three of the steers duplicate determinations of the percentage are remarkably constant, i. e., 33.6 and 34 for steer 1, 29.2 and 29.7 for steer 2, and 26.8 and 26.7 for steer 3. For steer 4, two of the triplicate determinations are in excellent agreement, 33.4 and 32.7, although the third determination was appreciably smaller, i. e., 30.9. Considering the fact that the duplicate insensible loss determinations, except for the last two on steer 4, were made at intervals ranging from 2 weeks to 3 months, while the intervals between respiration tests for the same steer were nearly the same or longer, this evidence of individuality seems decisive.

It is interesting to note that the steers exhibiting the highest percentages of heat emitted by the vaporization of water, steers 1 and 4, are the steers that metabolized the greater proportions of the gross energy of the feed (table 5) due to a more efficient digestion of the feed. Also, the steer emitting the smallest portion of heat in

vaporized water was almost continuously bloated and excreted greater than average amounts of methane. Whether these relations possess a causal significance cannot at present be determined.

Evidently the best prediction of the heat production of steers from the insensible loss in body weight cannot be made from any average relationship between the two, but should be based upon a relationship determined in preliminary experiments upon each individual steer. With this fact in mind, different methods of making this prediction may now be considered.

The heat production may be very accurately predicted from equation (15) of footnote 4

$$h = \frac{ei}{1.7241ce + 1.9769(r. q.) - 1.4290}$$

but this prediction would require for each steer a knowledge, not only of  $c$ , the percentage of heat lost by the evaporation of water, and of  $i$ , the insensible loss in body weight, but also of the respiratory quotient and of  $e$ , the heat equivalent of a liter of oxygen. If the respiratory quotient is less than 1,  $e$  may be computed from it, but if the respiratory quotient is above 1, as it will be on production rations, no rational method for the computation of  $e$  is at hand.

If the respiratory quotient for a given steer, the ration, and the level of feeding are known and if the respiratory quotient is above 1, the heat value of a liter of oxygen may be roughly estimated from the respiratory quotient by the following linear equation, describing the relationship observed between the two values in the series of respiration experiments reported in table 7:

$$e = 1.242(r. q.) + 3.744$$

The methane production may be computed with sufficient accuracy from the estimated digestible carbohydrates consumed by using the factor of Armsby (1) of 4.5 g of  $\text{CH}_4$  per 100 g of digestible carbohydrates. In the predictions to be made below it has been estimated at 21.7 g per kilogram of ration, the average output observed in these experiments.

The first prediction method involves, therefore, the observed insensible loss in body weight ( $i$ ), the observed percentage of heat lost as vaporized water ( $c$ ), the observed respiratory quotient, and the estimated value of  $e$ , the heat value of a liter of oxygen. In columns 4 and 5 of table 9, the value of this method is assessed by predicting the heat productions of the steers in the various experimental periods as described, and noting the percentage error incurred as computed from the observed heat productions. The average error is only 1.15 and the individual errors do not exceed 3.02 percent, incurred in the first experimental period on steer 4. For this steer, the average value of  $c$  for all three periods was used.

TABLE 9.—*Estimation of heat production from insensible loss in body weight by various methods explained in the text*

Steer no	Experiment no.	Observed heat production <sup>1</sup>	Estimations of heat production							
			Method 1		Method 2		Method 3		Method 4	
			Heat production	Deviation from observed	Heat production	Deviation from observed	Heat production	Deviation from observed	Heat production	Deviation from observed
		Calories	Calories	Percent	Calories	Percent	Calories	Percent	Calories	Percent
1.	116-35	8,795	8,707	-1.00	8,715	-0.91	8,523	-3.09	8,853	+0.66
	119-35	12,391	12,479	+0.71	12,450	+0.48	12,046	-2.06	13,135	+6.00
2.	118-35	8,966	8,874	-1.03	8,857	-1.22	8,957	-0.10	9,340	+4.24
	122-35	10,106	10,141	+0.35	10,109	+0.03	10,341	+2.33	10,791	+6.78
3.	115-35	9,593	9,533	-0.63	9,596	+0.03	9,305	-3.00	9,741	+1.54
	121-35	10,163	10,210	+0.46	10,215	+0.51	10,028	-1.35	10,496	+3.28
4.	117-35	10,093	9,788	-3.02	9,756	-3.34	9,997	-0.95	10,399	+3.03
	120-35	10,736	11,027	+2.71	11,014	+2.59	11,038	+2.81	11,481	+6.94
	123-35	10,281	10,384	+1.00	10,374	+0.90	10,373	+0.90	10,790	+4.95
Average <sup>2</sup>				1.15		1.11		1.84		4.16

<sup>1</sup> Corrected as in table 8<sup>2</sup> Disregarding signs of deviations

A second prediction method would involve arbitrarily assigning a value of 5.047 calories to  $e$ , corresponding, at least in animals promoting no considerable gastrointestinal fermentations, to a respiratory quotient of 1. The other bases of prediction remain the same. From table 9 it appears that no loss in accuracy is incurred by assigning an arbitrary, though not unreasonable, value to  $e$ . In fact, since  $e$  occurs in both the denominator and the numerator of the prediction equation, it may be expected that even considerable errors in the value assigned to it would not lead to serious errors in the estimate of heat production.

In the third prediction method to be considered the average respiratory quotient observed for all steers, i. e., 1.070, will be used in place of the individually observed respiratory quotients, and  $e$  will be assigned a value of 5.073, estimated from this respiratory quotient by the equation given above. While the prediction errors are somewhat larger for this method than for the other two, averaging 1.84 percent as compared with 1.15 and 1.11 percent, the method still is satisfactory, since the individual errors do not exceed 3.09 percent.

However, if the respiratory quotient is arbitrarily assigned a value of 1, and the heat value of a liter of oxygen ( $e$ ) the corresponding value of 5.047 calories, the predictions become appreciably less accurate, the average percentage deviation from the observed heat productions being 4.16. The individual errors range up to 6.94 percent (table 9).

It is clear that the success of a method of predicting the heat production of steers from the insensible loss in body weight depends upon (1) a careful determination of the insensible loss under standardized conditions, (2) a preliminary estimate on each steer of the fraction of heat lost in the vaporization of water, any ration being suitable for this purpose, and (3) fairly good estimates of the respiratory quotients to be expected for the experimental rations. If the errors of

the latter estimates are not more than 0.05 or 0.06, excellent predictions may be expected, while even errors of 0.10 would not be expected to produce errors of estimate greater than 7 percent or so.

#### SUMMARY AND CONCLUSIONS

The percentage of the insensible loss in body weight represented by vaporized water varies inversely with the respiratory quotient and approaches 100 as the percentage of heat lost from the body by the vaporization of water increases.

The insensible loss in body weight of steers is sensitive to changes in environmental temperature, particularly above a temperature of 70° F. Changes in relative humidity of the surrounding air are without certain effect up to values of 80 percent or more.

The hair coat of a steer is a potent factor in determining the insensible loss in body weight through its effect upon the vaporization of water from the skin. Removal of most of the hair coat by shearing may lower the insensible loss of weight by 20 to 30 percent. The shearing of steers tends markedly to equalize the percentages of heat lost through the vaporization of water.

A regular intake of water favors a regular insensible loss in weight. Abstinence from water for several days may depress the insensible loss in weight to abnormally low levels.

With rigorous control of all conditions with reference to the environment and to the animal that are known to affect the vaporization of water, the day-to-day variation in the insensible loss of body weight of steers confined in stalls may be reduced to a magnitude represented by a coefficient of variation of about 6 percent.

The ratio between heat lost by the vaporization of water and total heat output is not constant under the same environmental conditions but is an individual characteristic, at least for steers in the sheared condition.

The heat production of steers on a series of experimental rations may be estimated with small error from the following data:

(1) A careful determination of the insensible loss in body weight on each ration over a period of 2 weeks (or possibly less) under standardized conditions, as defined above.

(2) A preliminary estimate on each steer of the fraction of heat lost in the vaporization of water under the same conditions to be used in the subsequent experiments, any ration being suitable for this purpose.

(3) Fairly good estimates of the respiratory quotients to be expected on the various experimental rations. If the errors in these estimated quotients are not more than 0.05 or 0.06, excellent predictions of heat production may be expected, while even errors as great as 0.1 may not produce errors in estimated heat production of more than 7 percent.



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# VIABILITY OF CONIFER SEED AS AFFECTED BY SEED MOISTURE CONTENT AND KILN TEMPERATURE<sup>1</sup>

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## INTRODUCTION

In collecting conifer seed to use in reforestation, the cones must be harvested before the scales open or the seed will drop out and be lost. After they are harvested, the green cones must be thoroughly air-dried before the scales will open sufficiently to release all of the seed. In some regions weather conditions may interfere with drying operations. This condition and the development of the forest-seed industry have brought into use the dry kiln with its wide range of temperatures as a more controllable means of opening freshly gathered, moist cones. Kiln operation in the drying of cones cannot, however, be governed by the sole aim of completing the run in the shortest possible time. Many organic compounds are more liable to be disorganized by heat when they have a high moisture content than when they are dry, and seeds may be expected to react in this way. That in some seeds disorganization from the application of heat is greatest when moisture content is high is well known, even from the rather meager specific data on the subject. Obviously, the kiln operator must know what temperatures he can apply without injuring the moist seed. Also, he should know whether he may not apply much higher temperatures after part of the cone and seed moisture has been driven off by moderate heat. The object of the study discussed here was to determine, for certain conifer seeds, what effect a gradual increase in heat may have on moist seed, and the relation of such effects to increase in the moisture content of the seed. Furthermore, it was desired to determine the critical combinations of heat, moisture, and duration of heating. Tests were made with seed already extracted by air-drying. Results from such tests, although not exactly comparable with those in actual practice, were regarded as clearly indicative of results to be expected in seed extraction.

## REVIEW OF LITERATURE

The fact that certain dry seeds withstand higher temperatures than moist seed was reported by Eduard Heiden in 1859, Julius Sachs in 1865, and L. Just in 1875, according to citations by Atanasoff and Johnson (1).<sup>3</sup> Waggoner (14) later heated radish seed under a large number of different moisture content and temperature conditions and found also that the ability of the seeds to withstand heating varied inversely with their moisture content. For example, when seed of the Icicle radish, having a moisture content of more than 38

<sup>1</sup> Received for publication Nov. 20, 1935, issued July 1936.

<sup>2</sup> The experimental work for this report was done at the University of California, Division of Forestry. The writer received many helpful suggestions from F. S. Baker, A. W. Sampson, and A. R. Davis.

<sup>3</sup> Reference is made by number (italic) to Literature Cited, p. 864.

percent, was heated at 55° C. for one-half hour, the germination was subnormal, but when the seed-moisture content was only 0.4 percent, the germination was normal even after the seed was heated at 105° C. At 100° and more, the germination became less with each step of moisture increase above 0.4 percent.

Hottes and Wilson (8) obtained a moisture series similar to Waggoner's by placing wheat on trays in the upper part of jars which had various concentrations of sulphuric acid in the bottom. Despite the molding of samples with high moisture content, the investigators found that samples having 10 percent moisture, after being heated for 1 hour at 90° C. in a closed container, failed to germinate, but when heated at 80°, germinated 100 percent. Samples with 12 percent moisture gave no germination after heating at 80°, but 76-percent germination after heating at 70°. Germinating methods and sampling were not described.

Somewhat similar though less conclusive results were obtained by Harrington and Crocker (6), Kienholz (10), and Hofmann (7).

A number of investigators have studied the lethal temperatures for seed having a moisture content in equilibrium with ordinary storage conditions, but for the purposes of this study their work is of little value, since they made no record of either the air- or seed-moisture content. These include Atanasoff and Johnson (1), Burgess (2), Ewart (3), Gaine (5), Jodin (9), Robertson (11), Spafford (12), Staker (13), and Wright.<sup>4</sup> From these investigations dealing with lethal temperatures for seed of no stated moisture content, one can only conclude that with alfalfa, barley, wheat, oats, and acacia there is likely to be a decrease in germination after the seed has been subjected to about 90° C. for several hours, but seed from Douglas fir, ponderosa pine, peas, and cress evidently is injured at a lower temperature.

## EXPERIMENTAL METHODS

### MATERIAL USED AND NATURE OF TESTS

The seed used in this experiment was that of Douglas fir (*Pseudotsuga taxifolia* (LaMarck) Brit.) and ponderosa pine (*Pinus ponderosa*, Dougl.). It was purchased from a dealer in forest seed at Longview Wash., and was accompanied by declarations of origin. The fir seed came from an altitude of 700 feet in Cowlitz County, Wash. The pine seed was from Antelope Creek watershed, Siskiyou County, Calif., elevation 4,800 feet. Both kinds of seed were harvested in the fall of 1930 and had been extracted from air-dried cones.

The heating experiments were made in February 1931. The seeds were heated under 36 different combinations of conditions, including 3 known degrees of moisture, a long and a short period of heating, and 6 temperatures ranging by 5-degree intervals from 45° to 70° C. The percentage of germination from 5 samples, each containing 100 seeds, was used as the criterion of the result of each different treatment. Thus, a total of 180 germination tests was made. To obtain a basis for the choice of procedure, the effect of small changes in conditions and the reliability of the germination percentage were measured in preliminary experiments.

<sup>4</sup> WRIGHT, E. THE VIABILITY OF NATIVE AND EXOTIC SEED AT HIGH TEMPERATURES. Berkeley, Calif. 1923. (Thesis, Univ Calif)

## ADJUSTING THE MOISTURE CONTENT

Three moisture conditions were chosen with the intention of having a maximum and minimum that are commonly encountered and an intermediate point to help determine any graduation of effects due to the moisture.

The driest sample came from seed stored in crocks under laboratory conditions, averaging  $7 \pm 0.5$  percent moisture. The intermediate moisture condition was set at 30 percent, which represents that of seed in cones that have been stored for some time previous to extraction. The maximum moisture condition was set at 60 percent for the fir and 50 percent for the pine, because moisture of these percents approaches the greatest amount of water which these seeds will absorb and approximates the moisture content of freshly gathered cones.

A moisture content of 30 percent was obtained by soaking the Douglas fir seed in water for 35 minutes and the ponderosa pine seed for 3 hours. The moisture contents of 60 percent for Douglas fir seed and 50 percent for the pine were obtained by soaking the fir seed for 10 hours and the pine seed for 15 hours.

After the water was drained from the seeds, they were spread on a paper towel for approximately 5 minutes to allow the superficial water to dry. The seed was mixed and respread several times during the drying period, so that it would have a uniform exposure. When the coats appeared free from a water film, the seed was put in stoppered heating bottles and a 6-g sample set aside for a moisture determination (based on the constant weight after drying the sample at  $90^{\circ}\text{C}.$ ) These checks showed that the seed classed as having 30 percent moisture actually had a range of  $\pm 2$  percent, and that classed at 50 or 60 percent actually had a range of  $\pm 3$  percent.

## HEATING

The seed was heated at temperatures of  $45^{\circ}$ ,  $50^{\circ}$ ,  $55^{\circ}$ ,  $60^{\circ}$ ,  $65^{\circ}$ , and  $70^{\circ}\text{C}.$  in the upper part of a thermostatically controlled electric oven. By means of a small motor-driven fan, the desired temperature was maintained in all parts of the upper half of the oven with a variation of only  $\pm 1^{\circ}$ .

The seed containers were glass bottles, the cork stoppers of which had a fine groove cut in the side to allow a balance with atmospheric pressure. The bottles containing the pine seed were 2 inches square and 4 inches tall, and those containing the Douglas fir seed were  $1\frac{1}{4}$ -inch round bottles, 3 inches tall.

It was desired to prevent any significant drying in the seeds during heating. Trial samples showed no appreciable loss in moisture content during a preliminary 10-hour heating period at  $50^{\circ}\text{C}.$ , and moisture-content samples taken in the regular heating periods indicated that 2 percent was the maximum loss to be expected, this occurring in the samples which had 30 percent or more moisture at the beginning.

About 1 hour was required for the temperature at the center of a bottle of dry seeds to equal an oven temperature of  $50^{\circ}\text{C}.$ ; therefore it was necessary to preheat the seed in a quick way. To do this, the glass containers were warmed, then the seed was introduced, and, with thermometers thrust through the stoppers, the bottles of seeds

were warmed in a small hot oven. The samples were constantly watched and shaken by hand so that uniform heating from the outside to the center would be obtained. The desired temperature generally was reached in 5 to 10 minutes, and the bottles were then quickly transferred to the main oven.

The thermometers in the bottles were allowed to project through the two ventilator holes in the top of the main oven and were checked frequently to avoid any accidental fall or rise in temperature. The temperatures within the bottles did not vary more than  $1^{\circ}$  C. Six bottles of seed usually were put in the oven at the same time so that seed representing three different moisture conditions and two different durations of heating were subjected to the same temperature.

To determine the effects of two different durations of heating, some of the bottles were removed from the oven at the end of half an hour, the rest remaining 3 hours. Upon removal, the seed was spread on paper towels to dry and cool for 24 hours, whereupon it was stored in unsealed envelopes for 1 week before the germination tests were made.

#### GERMINATION

Since it would be very difficult to measure the separate physiological changes within seed which had been heated, the ultimate effect of high temperature was judged by the percentage of germination after heating. To obtain a standard for comparison of effects, eight random samples of unheated seed taken from the same crock were germinated. To have these under representative sprouting conditions, four samples were placed in each of the two germinating chambers.

By means of a graduated test tube of small diameter, the seeds were measured into lots containing approximately 100 seeds each, and no accurate count was made until the end of the germination period. Each lot was put in a separate test tube to be soaked in water 24 hours, after which time the water was drained off and the sample was placed on an individual plate in the germination chamber.

The germination plates were rough-surface, 4- by 3-inch paper ice-cream plates that had been soaked in water for 3 weeks to make them thoroughly saturated when first put in the germinator.

The germinating equipment consisted of two automatically controlled, water-jacketed germinators, with eight wire shelves in a chamber 18 by 20 by 20 inches and a water-pan humidifier under the bottom shelf. Temperatures were maintained at  $27^{\circ}$  C. The atmosphere was saturated at all times to prevent excessive drying of the plates.

The samples were inspected at 5-day intervals by removing one shelf at a time and recording for each numbered plate the number of seeds having protruding sprouts. The recorded seeds were then discarded. If molds or midge fly larvae appeared on the plate, or if some of the radicles had started to wilt as a result of damping-off fungi, the observation was included in the notes, in order that any correlation between the presence of these associates and the seed viability might be discovered. If any of the plates needed moistening, they were sprayed with a fine jet of water.

The tests were continued for 60 days, whereupon it was estimated from cumulative germination curves that sprouting of viable seeds

was complete. Previous cutting tests had shown the original stock of pine seed to have from 7- to 30-percent culls. To determine accurately the germination percentage, it was necessary to know the number of fully developed seeds in each sample. This was done in the case of the ponderosa pine samples by cutting open the ungerminated seeds and eliminating the undeveloped specimens. Germination percentages were based upon the number of sprouted ponderosa pine seed plus the developed but unsprouted seed. From previous cutting examinations it was found that the original Douglas fir seed was 98-percent sound, and since most of the ungerminated Douglas fir seed in the sprouting tests had disintegrated before the end of 60 days, no careful cutting tests were made on them after germination, the original number in each sample being used as the basis for germination computations.

As the plates were inspected, the area of each plate covered by the black mold *Chaetomium elatum* was estimated in tenths; the ratio of seeds attacked by the white mold *Verticillium* sp. and the presence of midge larvae were recorded on a scale of 10; and the presence of crystals, as yet unidentified, noticed on some of the pine-seed coats, was indicated in the notes. Since the presence and amount of damping-off fungi, molds, fly larvae, and crystals later proved to have no correlation with the germination results, they will not be discussed further.

## RESULTS AND DISCUSSION

### ANALYSIS OF THE DATA

The first step in handling the data was to determine the final germination percentage of each of 360 samples, and, for each species, to average these percentages for the five samples which had the same moisture content and were heated at the same temperature for the same length of time. The standard deviation of the five samples for each treatment was computed and compared with the deviations of individual samples which occurred farthest from the average. None of the samples showed sufficient deviation from the mean to be rejected. The standard error (standard deviation of a mean) was also computed.<sup>5</sup> The final germination percent and the standard error of each are given in table 1.

After computing the final germination percentages, the cumulative germination percentage for each sample and the mean of the five duplicate samples was computed for the end of each 5-day period. These cumulative mean values are plotted in figures 1 and 2 to illustrate the progress of sprouting. The standard deviation of the five samples around the moving average formed by the cumulative mean values was computed for each curve and is shown in the figures.

To those who are accustomed to germinating agricultural crop seeds, the variations in the following tests may seem unusually large. However, it has been the experience of investigators using forest seed that tree seeds are more liable to give low germination percentages and highly variable results.

<sup>5</sup> By the formula, standard error =  $\frac{\sigma}{\sqrt{N-1}}$ .

TABLE 1.—Average germination<sup>1</sup> of Douglas fir and ponderosa pine seed of given moisture content heated at various temperatures for 0.5 and 3 hours

DOUGLAS FIR							
Heating tempera- ture (°C) and period	7 percent moisture content	30 per- cent moisture content	50 per- cent and 60 percent mois- ture content	Heating tempera- ture (°C) and period	7 percent moisture content	30 per- cent moisture content	50 per- cent and 60 percent mois- ture content
45°	<i>Percent</i>	<i>Percent</i>	<i>Percent</i>	60°	<i>Percent</i>	<i>Percent</i>	<i>Percent</i>
0 5 hour.....	62±3 2	68±1.8	<sup>2</sup> 58±3 0	0 5 hour.....	61±1 7	0	0
3 hours.....	65±2 2	<sup>2</sup> 55±3.8	<sup>2</sup> 38±3 6	3 hours.....	64±4.3	0	0
50°				65°			
0 5 hour.....	62±3 0	60±2.8	37±1 2	0 5 hour.....	<sup>2</sup> 43±1 9	0	0
3 hours.....	57±4.5	25±3 1	1±0 6	3 hours.....	53±2.0	0	0
55°				70°			
0 5 hour.....	66±1.5	<sup>2</sup> 30±3.9	0	0 5 hour.....	11±2.8	0	0
3 hours.....	<sup>2</sup> 52±3 6	1±0.2	0	3 hours.....	18±2.3	0	0
PONDEROSA PINE							
45°				60°			
0 5 hour.....	24±1 8	23±2.8	17±2.7	0 5 hour.....	18±1 5	2±0 5	4±0 8
3 hours.....	21±3 0	21±2.8	23±3 3	3 hours.....	21±1 8	0	0
50°				65°			
0 5 hour.....	21±1.3	25±1.8	<sup>2</sup> 18±2 4	0 5 hour.....	13±2.0	0	0
3 hours.....	22±2 2	19±2.8	<sup>2</sup> 9±1.4	3 hours.....	11± 9	0	0
55°				70°			
0 5 hour.....	<sup>2</sup> 19±2 2	<sup>2</sup> 16±3 5	17±1 9	0 5 hour.....	9±2 1	0	0
3 hours.....	<sup>2</sup> 17±2 1	<sup>2</sup> 3±0.6	0	3 hours.....	7± .7	0	0

<sup>1</sup> The significance of the mean is given in terms of the standard error of the mean. A range of 3 times the standard error above and below the mean of 5 observations includes, according to normal probability, 96 percent of all other means which might be obtained from additional samples of the same material (4).

<sup>2</sup> For the indicated seed moisture content and duration of heating, this value represents the first significant deviation from normal that resulted from increase in the temperature applied. The normal values were 68±1.8 percent for Douglas fir and 29±2.4 percent for ponderosa pine.

According to some authors, not only the final germination percentage at the end of a long period but also the germination that takes place within a short time should be considered as an indicator of the physiological condition of seed. Harrington and Crocker (6) found, in testing heated Kentucky bluegrass seed, that the germination, although it later equaled the normal, was subnormal during the early part of the germination period. To study such relationships for Douglas fir and ponderosa pine, the writer plotted germination percentages for 5-day intervals. From inspection of figures 1 and 2, it is apparent that the condition which Crocker and Harrington observed does not exist in the present experiment. In fact, it is reversed. Germination of Douglas fir seed heated while in a moist condition was, for the first 20 days, equal to that of the unheated samples. After 20 days the heated seed did not germinate at the same rate as the unheated samples. This is shown by the fan shape of the curves in figures 1, B, and 2, A. However, the pine-germination progress curves begin to show differences in seed viability by the fifth day and remain nearly parallel after that (figs. 1, C, and 2, B).

#### TOTAL GERMINATION RESULTS AND THEIR APPLICATION

In table 1 it is seen that dry (7-percent moisture content) Douglas fir seed was not injured by heating for 3 hours at 50° C., but if the moisture content was 30 percent, a great loss in germination occurred.



Also, dry seed heated at 60° germinated normally, but seed with 30 percent or more moisture content heated for one-half hour at the same temperature failed completely. A temperature of 50° is often used in seed-extraction kilns. If freshly gathered cones (which usually have a moisture content of much more than 30 percent) are put in a

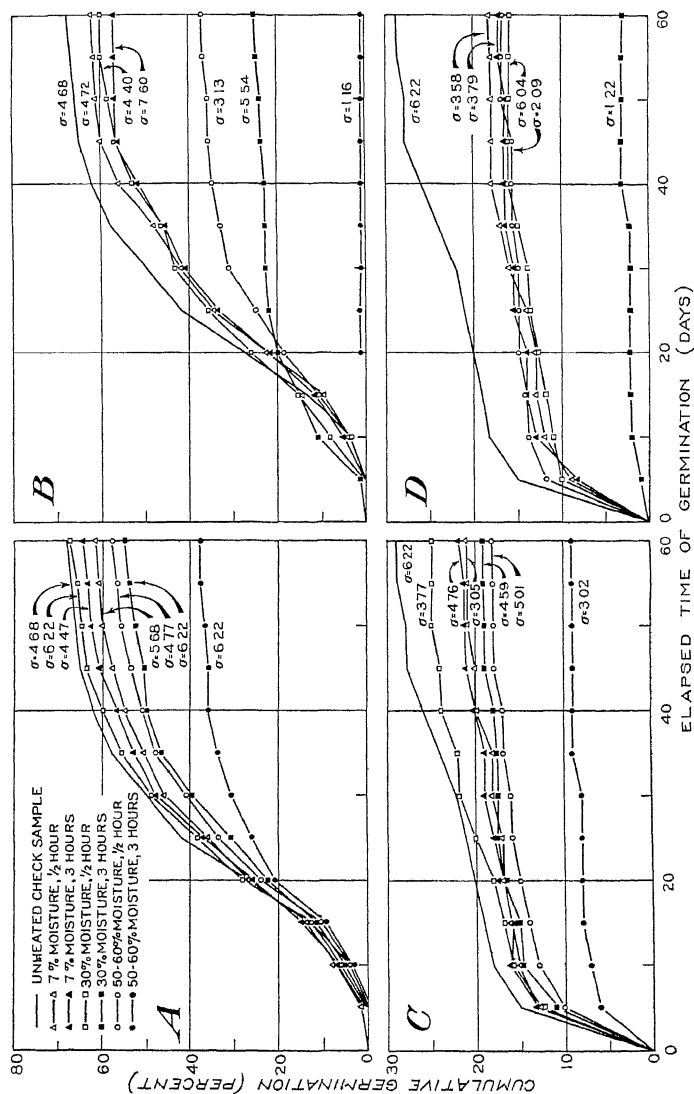


FIGURE 1 — Germination of seed of different moisture content under different degrees and periods of heating. A, Douglas fir seed heated at 45° C, and B, at 50° for one-half hour and for 3 hours; C, ponderosa pine seed heated for the same periods at 50°, and D, at 55°.

kiln at 50° and are not allowed free air circulation so that every cone with its seeds can dry out in less than 3 hours, probably the seed will be injured. If the moisture content is 60 percent when the cones are put in the kiln, the seed will probably be injured in less than one-half hour, unless it dries considerably within a few minutes.

It is possible that the relatively low temperatures which injure wet seed may reduce the germination of seed sown naturally. Surface soil temperatures often reach 50° C. in the fall when the soil and seeds are wet.

Table 1 shows that a temperature increase of 5° C. when heating Douglas fir or ponderosa pine seed may decrease the germination more than 30 percent. An increase from 50° to 55° C. caused a great loss

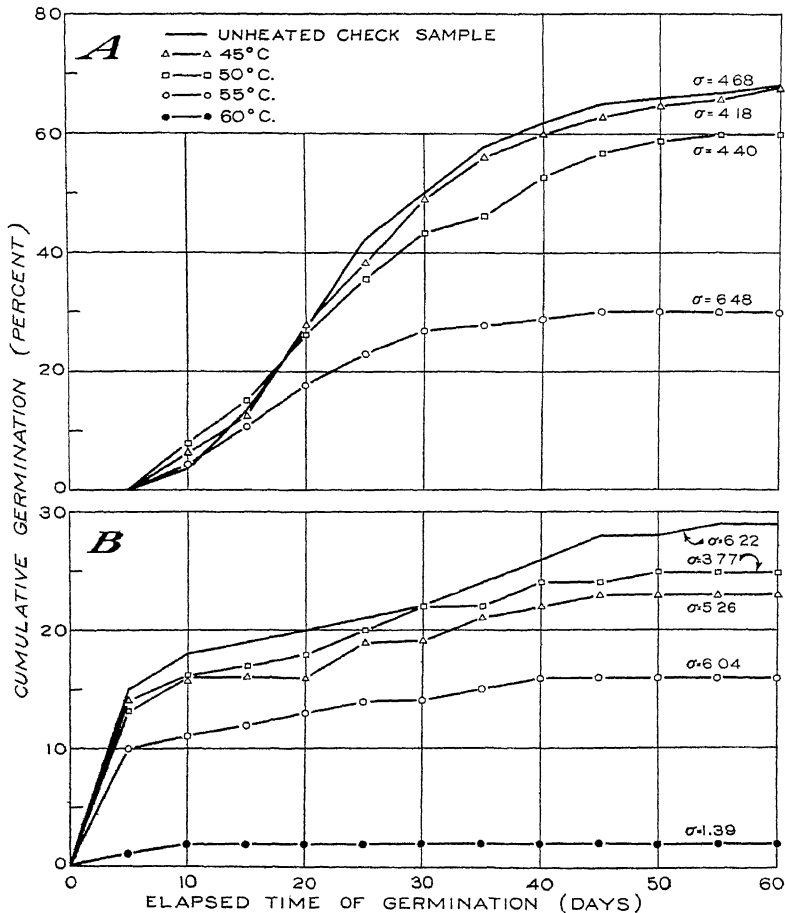


FIGURE 2.—Germination of seed which, at a moisture content of 30 percent, has been heated for one-half hour: A, Douglas fir; B, ponderosa pine.

in germination of Douglas fir seed when moisture content was 30 percent and the heating period was one-half hour, and a further increase of 5° caused a complete loss of viability. This indicates that care must be taken to keep all cones in a kiln at the same safe temperature. Temperatures at various places inside a drying chamber often differ by 5°. A temperature record taken at one point may indicate a safe condition while seeds at some other point are being destroyed.

The length of time that seeds are heated may have in some instances as great an effect on the viability as the seed-moisture content and heating temperature. When Douglas fir seed had a moisture content of 30 percent and was heated at 55° C., the germination after a ½-hour heating period was 30 percent, but after a 3-hour heating period was only 1 percent (table 1 and fig. 1, A and B). However, if the seed was dry, a 3-hour heating period had no appreciably greater effect than a ½-hour period. Apparently the heat resistance of dry seed is such that the initial damage is done in a short time, and no more injury is caused unless a very long heating period is used.

When dry Douglas fir or ponderosa pine seed (7-percent moisture content) is heated at 45° C. for one-half hour, the viability is not impaired. If the temperature, or seed moisture content, or time, or all three factors together, are increased unit by unit, there may be no evident effect on viability at first, but, ultimately, a combination of conditions is reached which causes a slight decrease in seed germination. This may be called a critical combination of conditions. Beyond that critical combination, any increase, either in heating temperature, seed-moisture content, or duration of heating, will cause a corresponding decrease in viability. It may be said that, in kiln drying, a slight increase in any one of these three factors is not dangerous unless the factors are already near a critical combination. But if cones are being dried near the critical heat point, the greatest care must be taken to avoid any increase in one of the three drying factors without a corresponding safe decrease in another factor. It will be of prime importance to the kiln operator to know the critical combinations of heating conditions. Within the range of conditions tested in this experiment, the critical points exist at some combination of conditions slightly more moderate than those which caused the first significant losses, as shown in table 2.

TABLE 2.—*Summary of temperatures causing first significant injury of seed at different moisture contents*

Seed-moisture content and duration of heating	Douglas fir	Ponderosa pine	Seed-moisture content and duration of heating	Douglas fir	Ponderosa pine
7 percent	° C.	° C.	50 percent (ponderosa pine):	° C.	° C.
½ hour.....	65	55	½ hour.....		50
3 hours.....	55	50-55	3 hours.....		45-50
30 percent.			60 percent (Douglas fir):		
½ hour.....	55	55	½ hour.....	45	
3 hours.....	45	50-55			

### SUMMARY

From the results of this experiment on seed of ponderosa pine and Douglas fir a few general statements may be made concerning the practice of kiln-drying cones of these species before shaking out the seed. It seems imperative that there should be a thorough circulation of drying air around every cone. The cones should not be in piles, as this will keep the inside ones moist. There will be economy in spreading the cones in a thin layer so that they may dry more rapidly, for the sooner they dry, the less apt they are to be damaged by heat.

The drying should be begun with a relatively low temperature—40° C. for instance—which may be increased as the cones become drier. Near the end of the run the temperature probably can be

raised to 55° if the seed-moisture content has already been reduced to 20 percent or less. If the cones have been somewhat air-dried before they are put in the kiln, they may be started at a higher temperature than more moist cones. Moisture-content samples should be taken, and the sacks of cones should be graded according to moisture content, if the greatest efficiency of kiln operation is desired. When this is done, a batch of wet cones can be run at a low safe temperature and a charge of dry cones at a faster-drying but equally safe temperature. In that way it will not be necessary to extend the heating period of the dry cones in order to prevent injury to the seed of a few moist cones.

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# THE COMPARATIVE INSECTICIDAL EFFICIENCY AGAINST THE CAMPHOR SCALE OF SPRAY OILS WITH DIFFERENT UNSULPHONATABLE RESIDUES<sup>1</sup>

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## INTRODUCTION

The percentage of the more chemically active constituents in a given mineral oil, as indexed by the unsulphonatable-residue test recommended by Graham (5, 6)<sup>3</sup> may indicate the approximate content of olefines or other unsaturated hydrocarbons, including aromatic, naphthenic, and resinous substances, as well as branched-chain paraffin compounds of high molecular weight. The large number of makes and grades of spray oils of the lubricating or engine oil type employed at present, together with the uncertainty about the chemical composition of each oil, prevents the making of a trustworthy comparison of the relative toxic effects of the above-mentioned active constituents on scale insects. However, although the different active constituents cannot be separated by the sulphonation test, it is possible to determine (by experiments) whether the more active compounds, as so measured collectively, have any effect upon the insecticidal action of the oils. Swingle and Snapp (8) have obtained data regarding the effect of several oils having different percentages of sulphonatable constituents, on the control of the San Jose scale (*Aspidiotus perniciosus* Comst.) on peach trees at Fort Valley, Ga., and report that wide variations in the content of sulphonatable materials produced little difference in toxicity to that insect. Their tests were conducted with oils containing sulphonatable portions ranging between 100 and 31.2 percent by volume. On the other hand, English (4) states that under some conditions the unsaturated oils may be more toxic.

Since any investigation of the toxicity of the sulphonatable constituents must be made with oils of both unknown and differing composition, it should be borne in mind that the results obtained in using a small number of oils are not necessarily applicable to all such oils. With recognition of this limitation, the laboratory and field experiments described below were planned. The objective was to determine whether or not three mineral oils containing markedly different percentages of sulphonatable material would produce measurably different mortalities of the camphor scale, *Pseudaulnobia duplex* (Ckll.), at New Orleans, La. Spuler, Overley, and Green (?) report that the insecticidal value increases with the viscosity of oils; and, if such is the case, the effects of the sulphonatable portions, provided they influence the insect, should be separated from effects attributable

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<sup>2</sup> Authors' names are given in alphabetical order; the sequence indicates no seniority.

<sup>3</sup> Reference is made by number (italic) to Literature Cited, p. 878

to viscosity. Hence oils were selected which had substantially the same viscosity and volatility in order that the effect of variations in these two properties would not obscure differences in results due to variations in the sulphonatable content.

## EXPERIMENTAL METHODS

### SPECIFICATIONS OF OILS

All sprays were prepared from two oils purchased from a well-known manufacturer and designated for experimental purposes as WR and L-1778. The Saybolt viscosities, the percentages of non-volatile and of sulphonatable portions, and the specific gravities were separately determined and are presented in table 1.

TABLE 1.—*Characteristics of oils used in experiments with sprays against the camphor scale, New Orleans, La., 1932*

Oil designation	Saybolt viscosity at 100° F.	Nonvolatile material (24 hours at 100° F.) <sup>1</sup>	Sulphonatable material (by volume)	Specific gravity at 68.0° F.
	<i>Seconds</i>	<i>Percent</i>	<i>Percent</i>	
WR.....	94.9	98.6	6	0.8512
WR/L-1778.....	97.8	98.6	16	.8679
L-1778.....	101.9	98.4	33	.8846

<sup>1</sup> Evaporation tests were made according to methods proposed by Dawsey (2).

Distillation ranges were not determined, but according to the manufacturer's specifications the WR oil began distillation at 654° F. with 89 percent off at 750°, and the L-1778 began distillation at 630° with 87 percent off at 750°. The L-1778 was stated to be a blend of two or more straight-run oils. The oil designated as WR/L-1778 consisted of equal parts of the first and third oils, compounded by mixing them before preparing emulsions therefrom.

### PREPARATION OF EMULSIONS

The emulsions consisted of the mineral oils dispersed in an aqueous solution of sodium oleate. Concentrated emulsions containing 66½ percent of oil by volume were made according to the process suggested by Dawsey,<sup>4</sup> wherein emulsification takes place spontaneously and the emulsifying soap is formed simultaneously. These emulsions were then usually diluted to an intermediate concentration of 40-percent oil before the highly diluted sprays containing from 0.9 to 2 percent of oil were prepared. The stock emulsions used throughout the work were numbered consecutively from 1 to 9, inclusive, and the sprays made from them were designated in a like manner.

Table 2 shows the composition of the sprays applied to potted camphor-tree (*Cinnamomum camphora*) plants in the laboratory. The stock mixtures were prepared 1 day prior to application of the sprays, the sprays being made up at the time of application by dilution with distilled water.

<sup>4</sup> DAWSEY, L. H. SPONTANEOUS EMULSIFICATION IN THE PREPARATION OF OIL SPRAYS. Unpublished manuscript

TABLE 2.—Composition of oil sprays used against the camphor scale on potted camphor-tree plants in the laboratory June 14 and Aug. 24, 1932

Stock emulsion no. <sup>1</sup>	Oil used	Date of spraying	Composition of sprays			
			Spray no. <sup>2</sup>	Volume of stock emulsion taken	Oil content of spray	Normality of sodium oleate in aqueous phase $\times 10^4$
				<i>Cc</i>	<i>Percent</i>	
1.....	WR.....	June 14	f1a.....	50	1.0	6.62
			f1b.....	85	1.7	11.34
2.....	WR/L-1778.....	do.....	f2a.....	50	1.0	6.62
			f2b.....	85	1.7	11.34
3.....	L-1778.....	do.....	f3a.....	50	1.0	6.62
			f3b.....	85	1.7	11.34
4.....	WR.....	Aug. 24	f4a.....	45	.9	5.95
			f4b.....	75	1.5	9.98
5.....	WR/L-1778.....	do.....	f5a.....	45	.9	5.95
			f5b.....	75	1.5	9.98
6.....	L-1778.....	do.....	f6a.....	45	.9	5.95
			f6b.....	75	1.5	9.98

<sup>1</sup> Each stock emulsion was made of 200 cc of oil, 4.3 cc of 3.05N oleic acid, and 300 cc of distilled water, and had an oil content of 40 percent by volume.

<sup>2</sup> The total volume of the spray after dilution was 2,000 cc

For the field sprays, both the oil concentration and the soap concentration were higher than in the laboratory sprays. The oil concentration was 2 percent and the emulsifier concentration (normality of sodium oleate in the aqueous phase) was  $24.7 \times 10^{-4}$ . The effect of increasing the oil content is to deposit a heavier film of oil on the tree. The effect of increasing the soap concentration is to decrease the oil deposit, but in this particular case, where 100 gallons of spray was made at a time, it was necessary to use New Orleans tap water, and an excess of soap was necessary to overcome its hardness and at the same time keep the oil globules in suspension.

Field-spray experiments (1) conducted in 1931 had shown that the kills most suitable for accurate mortality determinations (50 to 75 percent) might be obtained by using a spray containing 2 percent of oil (emulsified with potassium fish-oil soap) and having a soap normality of  $26.3 \times 10^{-4}$ . Effort was therefore made to approximate that composition in the field sprays. For each stock emulsion the oil was emulsified by the spontaneous method into a mixture containing 40 percent of oil. Two gallons of the oil was poured into a 15-gallon oak keg, to which a stirrer driven by a  $\frac{1}{4}$ -horsepower, 1,750-revolutions per minute motor was clamped; 0.0793 gallon (300 cc) of 3.05 normal oleic acid was stirred into the oil; 0.0817 pound of C. P. sodium hydroxide was dissolved in 1 gallon of distilled water, and this alkali solution was slowly added to the oleic acid-mineral oil mixture with fast mixing. After emulsification, 2 gallons more of distilled water was added and the whole 5 gallons of emulsion stored overnight in a large milk can for use in field spraying the following day.

These stock emulsions, nos. 7, 8, and 9, each containing 40 percent of oil (WR, WR/L-1778, and L-1778, respectively), were diluted with tap water to a final volume of 200 gallons to form sprays 7F, 8F, and 9F, each containing 2 percent of oil. The emulsifier concentration

(normality of sodium oleate in the aqueous phase) of  $24.7 \times 10^{-4}$  includes oleates of calcium, magnesium, etc., formed with undetermined quantities of mineral salts in the dilution water.

#### APPLICATION OF LABORATORY SPRAYS

The potted camphor-tree plants to which the laboratory sprays were applied had previously been artificially infested by the "box" method (1); so the populations were of uniform age distribution, and differences in the mortality of scales attributable to variation in age susceptibility were eliminated. The insects were in the adult stage at the time of spraying.

The laboratory spraying outfit consisted essentially of apparatus already described, and standardized conditions of spray application, like those described in the earlier paper (1), were maintained. The applications were made with a compressed-air sprayer consisting of a 2-liter metal tank, to which was attached a short piece of rubber hose fitted with a vermorel nozzle. The air-inlet pipe reached nearly to the bottom of the tank, so the incoming air provided sufficient agitation to insure an even distribution of oil within the tank. The infested plants were placed upon a revolving platform, at a distance of 1 meter from the nozzle, and each plant was sprayed for 24 seconds at a constant pressure of 40 pounds per square inch. The six sprays of the first set, numbered 1a to 3b (table 2), and containing 1 and 1.7 percent of oil, respectively, were applied on June 14. The other six sprays, designated 4a to 6b, were applied on August 24, but the oil concentrations were reduced to 0.9 and 1.5 percent, respectively, for the two series of the second set, because the scales of this brood were not so old as those of the earlier brood. Each set was treated as an independent experiment, since the applications were made at different times of the year. The number of plants composing the June 14 set was 46, of which 8 were sprayed with distilled water as checks; the number of plants composing the August 24 set was 42, of which 7 were sprayed with water as checks. The plants were from 15 to 20 inches in height and about 2 years old when sprayed.

#### APPLICATION OF FIELD SPRAYS

In the field sprays an entirely different technique was employed, the applications being made with a power sprayer of a type used in commercial work. The camphor-trees to be sprayed were located in Metairie Cemetery on both sides of a driveway running southwest and northeast. They were heavily infested with adult scales and ranged from 10 to about 25 feet in height. All were in the open and exposed to the sun at all times of the day. The trees along the drive were numbered from 1 to 10, going in the northeast direction, the numbers alternating from one side of the road to the other. Nos. 1, 6, and 9 were designated as check trees and were left unsprayed. The remaining seven trees were sprayed between 9 a. m. and noon on November 22. Spray 7F was applied on trees 2, 3, and 5; spray 8F on trees 4 and 7; and spray 9F on trees 8 and 10. Applications were



made with a power spray machine at 350 pounds' pressure. The time during which each tree was sprayed depended upon the size of the tree, and an attempt was made to secure uniform coverage or wetting of all parts of each tree. At the time of spraying a number (from two to five) of small branches on each tree were tagged as suitable for making counts and mortality determinations of the scales located thereon.

In order to determine whether any separation of the oil occurred in the spray tanks, samples were taken from the nozzle at the beginning and end of the application of each spraying and analyzed for oil content. These samples showed from 1.9 to 2 percent of oil. Since a difference of 0.1 percent is within the limits of accuracy of the method of sampling and analysis, it was concluded that no breakdown of the emulsions occurred while spraying, and that all trees received substantially the correct oil concentration.

## RESULTS

### OIL DEPOSITED BY SPRAYS

Since the previous work done in a study of the insecticidal action of emulsions at this laboratory (1) has shown that the percentage of insects killed is closely related to the quantity of oil deposited on the plant surface by the spray, analyses were made to determine the quantity of oil deposited on camphor-tree foliage by all sprays described herein.

At the time the potted plants were treated on June 14, portions of the same sprays were applied to pairs of branches from green camphor-trees. The number of leaves on the branches usually amounted to about 1,000. After being sprayed, the branches were allowed to dry, and from each branch (there were duplicate branches for each spray) approximately 500 leaves were clipped. These leaves were thoroughly mixed, and from each lot 200 disks 2 cm in diameter were stamped by means of a leaf die. The disk samples were then extracted with ether as the first step in the determination <sup>5</sup> (3) of the quantity of oil deposited by the respective sprays.

In the laboratory tests of August 24 the method of application of sprays to foliage more closely approximated the method by which the potted plants were sprayed. Branches cut from trees in the field were brought to the laboratory, and the smaller twigs were cut off and formed into bunches, which were then stuck into pots to simulate the potted plants. These were placed on the revolving platform and sprayed in the same way as the plants, except that the time of application was lengthened to 45 seconds because of the greater quantity of foliage. Table 3 shows the average volume of oil found per square centimeter of foliage, listed together with the sprays producing the deposit. (See table 1 for composition of these sprays.)

<sup>5</sup> DAWSEY, L. H. THE DETERMINATION OF UNREFINED MINERAL OILS RETAINED BY LEAF SURFACES FOLLOWING SPRAYING. Unpublished manuscript.

TABLE 3.—Quantities of oil deposited on camphor-tree foliage by laboratory sprays, showing analyses of duplicate samples for each spray

Sprays applied June 14, 1932				Sprays applied Aug. 24, 1932			
Spray no.	Analysis sample no.	Volume of oil deposited per square centimeter of leaf surface at 20° C.		Spray no.	Analysis sample no.	Volume of oil deposited per square centimeter of leaf surface at 20° C.	
		Volume found	Average volume			Volume found	Average volume
		$Cc \times 10^3$	$Cc \times 10^3$			$Cc \times 10^3$	$Cc \times 10^3$
1a.....	{ 3	4.59	4.4	4a.....	{ 3	4.04	4.1
	{ 4	4.22			{ 4	4.09	
2a.....	{ 15	4.22		5a.....	{ 15	4.17	
	{ 16	4.36	4.3		{ 16	4.04	4.1
3a.....	{ 9	4.20		6a.....	{ 9	3.77	
	{ 10	4.19			{ 10	3.77	
1b.....	{ 5	6.04	6.7	4b.....	{ 5	5.74	5.7
	{ 6	7.26			{ 6	5.59	
2b.....	{ 17	5.58		5b.....	{ 17	5.74	
	{ 18	5.55	5.6		{ 18	5.69	5.7
3b.....	{ 11	6.18		6b.....	{ 11	4.85	
	{ 12	6.12			{ 12	4.79	

The deposits produced by sprays 1a to 3a should be slightly greater than those of sprays 4a to 6a, inasmuch as the oil concentration in the former was 1 percent while in the latter it was only 0.9 percent. This appears generally to be the case for corresponding preparations in the sprays applied June 14 and those applied August 24. On the other hand, the average oil deposits of the "b" sprays, in each series, were higher than those of the "a" sprays in the same series, because the oil concentrations were much higher.

The disagreement between deposits from the sprays of equal strength, as shown in table 3, is not thought to be due to large errors in chemical analyses, since check samples containing known quantities of each oil were run simultaneously with the test samples. The lack of agreement is more likely to have been caused by nonuniform spraying of the duplicate tree branches, in spite of the precautions used in applying the sprays. A second source of error probably lay in the random difference in leaf surface as it affected the quantities of oil deposited. The question of lack of uniformity in coverage as being due to changing oil concentrations in the sprays during application was separately investigated for the laboratory sprays applied August 24. Samples of stock mixtures 4a to 6b (table 2) were placed in the reservoir of the spray apparatus, and the same volumes of spray liquids as were applied to each infested group of camphor plants were sprayed from the nozzle and caught in beakers for determination of the percentage of oil in each portion. There were six 300-cc portions in each case, including the liquid ejected at the beginning when the spray tank was full and the liquid at the end when the tank was nearly empty. If the oil globules actually had collected toward the top of the tank, the first portions of the spray would have been lower in oil content than the last portions, since the outlet pipe projected to the bottom of the spray reservoir. All tests made in this manner showed no change in the percentage of oil in the liquids, hence the trouble could not be located at this point. On the contrary, the oil-percentage determinations indicated that the quantity of oil

in the sprays as they left the nozzle of the apparatus could be controlled with high precision.

In preparation for the determination of the oil deposits produced by the field sprays, certain portions of each sprayed tree that offered the proper degree of infestation had been tagged prior to the spray application. Since mortality counts were to be made on these twigs, leaves from these twigs and from the immediate vicinity were detached after the sprays had dried, and disks 2 cm in diameter were cut from them. Duplicate samples of only 150 disks were taken from each tree, since the oil deposits on the foliage appeared to be heavy. The tagged, infested twigs, upon which the tips and younger shoots were left untouched, were left growing on the tree. The quantities of oil found in these samples are shown in table 4. Again, in these field sprays, differences in samples were found, but they are likewise attributed to small errors in analyses or to larger and unavoidable discrepancies due to nonuniform spray coverage.

TABLE 4.—Quantities of oil deposited on camphor-tree foliage per square centimeter of leaf surface in field spraying,<sup>1</sup> as shown by analyses of duplicate samples from each tree sprayed

Tree no.	Spray 7F	Spray 8F	Spray 9F	Tree no.	Spray 7F	Spray 8F	Spray 9F
	Cc $\times 10^5$	Cc $\times 10^5$	Cc $\times 10^5$		Cc $\times 10^5$	Cc $\times 10^5$	Cc $\times 10^5$
2.....	9.6	-----	-----	7.....	-----	10.2	-----
3.....	9.8	-----	-----	8.....	-----	9.4	-----
3.....	10.6	-----	-----	10.....	-----	-----	10.2
3.....	9.0	-----	-----	10.....	-----	-----	9.6
4.....	-----	9.4	-----	10.....	-----	-----	10.1
4.....	-----	8.9	-----	10.....	-----	-----	10.6
5.....	8.7	-----	-----				
5.....	10.4	-----	-----				

<sup>1</sup> Each spray contained 2 percent of oil. The soap concentration (normality of soap in the aqueous phase) of  $24.7 \times 10^{-4}$  includes oleates formed with sodium, calcium, magnesium, etc., in the dilution water.

#### DETERMINATION OF MORTALITY

Because of unavoidable delays in securing the proper oils, the scales at the time (June 14) of the first laboratory tests were reproducing by the time they were sprayed. If they had been kept under summer temperatures, many of the living scales would have deposited all their eggs and died before the mortality counts could have been completed, so it was necessary to retard the rate of development by subjecting the insects to low temperatures. The sprayed plants were placed in a cold room, where they were held at temperatures of 11° to 13° C. until the mortality had been determined. Counts were started June 29 and completed July 2, 18 days after spraying.

Earlier tests had shown that even lower temperatures did not cause any mortality of camphor scales when kept in the cold room for 3 weeks. In these preliminary cold-room tests, made earlier in the year, infested twigs were taken from camphor-trees in the field, the leaves removed, and the twigs (with the cut ends in water) were placed in the cold room, which was held at a temperature of 8° to 11° C. The natural mortality for one sample of these twigs was determined at the time of cutting, and for two other samples 15 and 21 days later. The results are given in table 5.

TABLE 5.—Proportion of adult scales found alive on sample twigs on different dates, 1932

Date counted	Period in cold storage	Overwintering adults counted	Adults alive
	Days	Number	Percent
Jan. 14.....	0	800	86.8
Jan. 29.....	15	779	84.2
Feb. 4.....	21	811	86.7

No significant changes in mortality were found; in fact, the counts made January 14 and February 4 showed unusually close agreement for duplicate samples from the field.

The counts were made in the manner described in a previous paper (1), but the methods of analyzing the data differed from those used

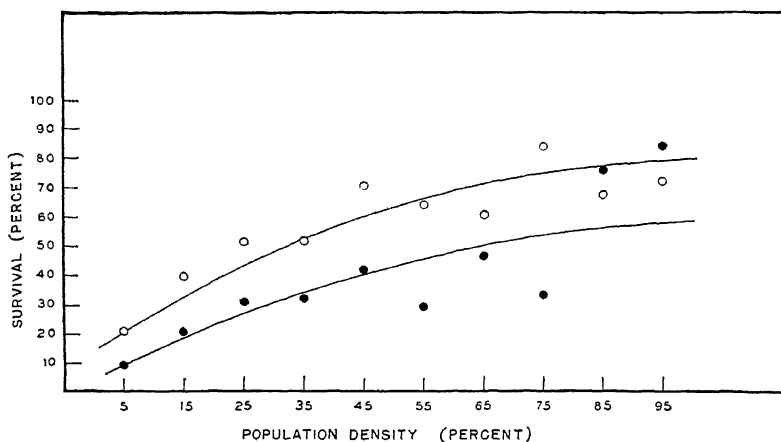


FIGURE 1.—Survival of camphor scales on potted camphor-tree plants sprayed June 14, 1932. The upper curve represents the average survival for the 1a, 2a, and 3a sprays, which contained 1 percent of oil. The lower curve represents the average survival for the 1b, 2b, and 3b sprays, containing 1.7 percent of oil. Open circles represent the average survival at different population densities for the 1-percent sprays; solid circles, the average survival for the 1.7-percent sprays.

in the earlier work. After determining the natural mortality at each population-density interval, the numbers of dead and living scales at each density were totaled for the plants sprayed with the same oil concentration. Corrections were made for natural mortality, and the percentages killed by the sprays estimated. In this way curves were obtained (fig. 1) which represented the average survival for the three oils at each oil concentration. Then the number surviving each oil was compared with the average survival for the three oils at the corresponding concentration, and the deviation from the average curve found at each density interval. To illustrate this procedure, a sample summary is shown in table 6. By taking the algebraic sum of the deviations, the differences in survival for the three oils were found. These differences were finally expressed as percentages of the total number of scales alive before spraying, as shown in table 7.

TABLE 6.—*Sample summary used in the estimation of relative effectiveness of sprays made from oils differing in sulphonatable content*

[The number of scales alive before spraying was obtained by multiplying the total number of scales at each population density by the percent alive in the check. The number expected to survive was estimated by multiplying the number alive before spraying by the average rate of survival at the corresponding density as shown in fig. 1. The data given are for spray 1a, containing 1 percent of oil.]

Population density	Scales alive before spraying	Scales surviving	Scales expected to survive	Deviation	Population density	Scales alive before spraying	Scales surviving	Scales expected to survive	Deviation
	Number	Number	Number	Number		Number	Number	Number	Number
5 percent----	82	14	17	-3	55 percent----	96	57	64	-7
15 percent----	113	45	37	8	85 percent----	50	31	39	-8
25 percent----	104	53	46	7	95 percent----	92	73	74	-1
35 percent----	82	46	43	3					
45 percent----	146	102	88	14					

TABLE 7.—*Comparison of rates of survival of camphor scales on potted camphor-tree plants treated with sprays made from oils differing in sulphonatable content; sprays applied June 14, 1932*

Spray no.	Proportion of oil sulphonatable	Oil concentration in spray	Average oil deposit per square centimeter of leaf surface	Plants	Scales alive before spraying	Deviation from average survival curve	
	Percent	Percent	Cc $\times 10^5$			In scales surviving <sup>1</sup>	In rate of survival
1a-----	6	1.0	4.4	6	765	+13	+1.7
2a-----	16	1.0	4.3	6	504	+19	+3.8
3a-----	33	1.0	4.2	7	662	-18	-2.7
1b-----	6	1.7	6.7	6	671	+2	+3
2b-----	16	1.7	5.6	6	557	-26	-4.7
3b-----	33	1.7	6.2	7	582	+26	+4.5

<sup>1</sup> The curves of fig. 1 were fitted so as to make the sum of the residuals approximately zero. Since in calculating the residuals the deviations from the curves were weighted by the square root of the number of scales at each density, the sum of the actual deviations as shown in this column will not necessarily be zero.

The methods of counting and analysis of the results of the sprays applied August 24 were the same as described for the June 14 sprays. Figure 2 shows the average survival curves for each of the two oil

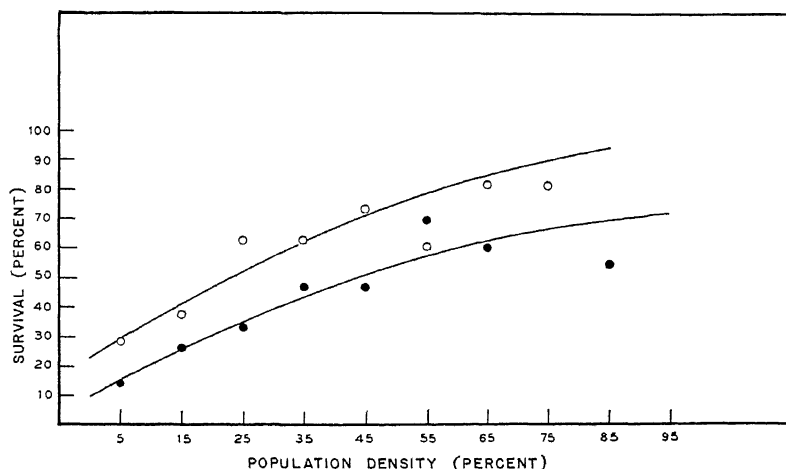


FIGURE 2.—Survival of camphor scales on potted camphor-tree plants sprayed August 24, 1932. The upper curve represents the average survival for the 4a, 5a, and 6a sprays containing 0.9 percent of oil. The lower curve represents the average survival for the 4b, 5b, and 6b sprays containing 1.5 percent of oil. Open circles represent the average survival for the 0.9-percent sprays, solid circles, the average survival for the 1.5-percent sprays.

concentrations. The deviations from these curves of the survival rates for each spray are given in table 8.

TABLE 8.—*Comparison of rates of survival of camphor scales on potted camphor-tree plants treated with sprays, made from oils differing in sulphonatable content; sprays applied Aug. 24, 1932*

Spray no	Proportion of oil sulphonatable	Oil concentration in spray	Average oil deposit per square centimeter of leaf surface	Plants	Scales alive before spraying	Deviation from average survival curve	
						In scales surviving	In rate of survival
	Percent	Percent	CcX10 <sup>3</sup>	Number	Number	Number	Percent
4a.....	6	0.9	4.1	5	<sup>1</sup> 281	-9	-3.2
5a.....	16	.9	4.1	5	697	+6	+9
6a.....	33	.9	3.8	6	839	-8	-1.0
4b.....	6	1.5	5.7	6	574	-42	-7.3
5b.....	16	1.5	5.7	5	1,093	+58	+5.3
6b.....	33	1.5	4.8	6	829	-22	-2.7

<sup>1</sup> Figures for this spray do not include 1 plant which showed only 21 percent dead (natural mortality in checks was 11.5 percent) and was inconsistent with the other 5 plants in this group. The reason for the low kill is unknown. Possibly the plant had a roughened bark instead of the usual smooth green bark. On such plants the kill is always lower. If this plant is included, the 4a spray shows a deviation from the average curve of 14.7 percent, the effect, therefore, would be to increase the variability of the results but not to indicate a difference due to the sulphonatable portion of the oil.

At the time the field sprays were applied the living overwintering scales were all adults, about 95 percent being in the pink and gravid stages. In making the counts from the field material the dead adults of the older broods and those attacked by predators or fungous diseases were classified separately. They were used in estimating the population density, but were then eliminated from further calculations. The proportion of dead immature scales was negligible, and such scales were not included in the counts.

Immediately before applying the sprays, infested branches were cut from the trees to be sprayed and the natural mortality was determined. No systematic variations in the proportion of dead scales were found throughout the rows. Counts were also made on branches of the check trees at intervals until the spray counts were completed, and no changes in the natural mortality were found during this time; accordingly, all the check counts were averaged to find the natural mortality at each population density. The proportion of dead scales in the unsprayed branches increased from 3 percent, when 5 percent of the twig area was covered by scales, to 8 percent when the population density was 95 percent.

From the natural mortality curve the number of scales estimated to be alive before spraying was found for each of the sprayed twigs, and from the number actually found alive the survival was calculated as a percentage of the number of scales alive before the application. The survival percentages are given in table 9 and are shown graphically in figure 3.

TABLE 9.—Comparison of the rates of survival of camphor scales (at different population densities) on camphor-trees in Metairie Cemetery, after treatment, Nov. 22, 1932, with sprays 7F, 8F, and 9F, made from oils containing different proportions of sulphonatable compounds, together with data concerning quantities of oil deposited on leaves and some of the properties of the oils used

## SPRAYS USED

Spray no <sup>1</sup>	Oil used	Saybolt viscosity of oil at 100° F.	Proportion of oil volatilized (24 hours at 100° F)	Specific gravity of oil at 68° F.	Proportion of oil sulphonatable	Average volume of oil deposited per square centimeter of leaf surface
		Seconds	Percent		Percent	CcX10 <sup>4</sup>
7F.....	WR.....	94.9	1.44	0.8512	6	9.7
8F.....	WR/L-1778.....	97.8	1.42	.8679	16	9.5
9F.....	L-1778.....	101.9	1.59	.8846	33	10.1

## RESULTS WITH VARIOUS SPRAYS

Population density (percentage of twig area covered by scales)	Checks		Spray 7F		Spray 8F		Spray 9F	
	Scales alive before spraying	Survival	Scales alive before spraying	Survival	Scales alive before spraying	Corrected survival	Scales alive before spraying	Corrected survival
	Number	Percent	Number	Percent	Number	Percent	Number	Percent
0-10.....	501	96.9	299	20.7	282	43.6	253	21.3
10-20.....	1,008	96.1	673	32.2	785	46.4	843	33.3
20-30.....	1,336	95.8	736	49.5	900	54.9	1,129	47.1
30-40.....	1,068	95.6	831	62.2	938	65.6	811	49.9
40-50.....	800	94.9	632	57.8	680	75.9	914	62.7
50-60.....	918	94.6	410	78.0	408	76.2	827	57.6
60-70.....	642	94.0	272	71.7	339	92.0	585	72.0
70-80.....	390	90.9	216	70.4	193	81.3	236	68.0
80-90.....	288	93.4	149	71.1	92	100.0	329	74.2
90-100.....	361	91.6	133	58.6	42	81.0	357	73.1

<sup>1</sup> Each spray contained 2 percent of oil to 98 percent of soap solution which was  $24.6 \times 10^{-4}$  normal in soaps.

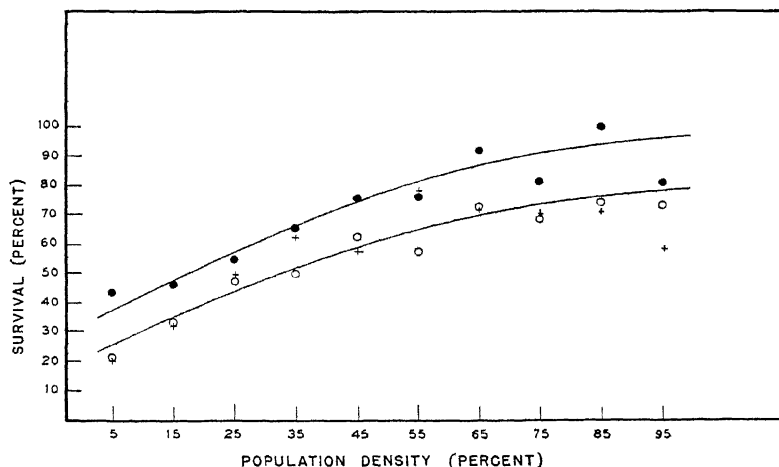


FIGURE 3.—Survival of camphor scales from oil sprays applied to camphor-trees. The lower curve represents the average survival for sprays 7F (WR oil) and 9F (L-1778 oil); the upper curve, the survival for spray 8F (containing a mixture of the two oils). Crosses represent survival from the WR oil spray; open circles, survival from the L-1778 oil spray; and solid circles, survival from the spray of the two oils mixed. Each spray contained 2 percent of oil.

From the curves of figure 3 it may be seen that the values at each density for the 7F and 9F sprays fell so close together that they could be fitted by one curve. The oils used in these sprays represented the two extremes in sulphonatable content. The survival from the 8F spray was uniformly higher over the entire range of population density. This oil was a mixture of the two other oils, and therefore intermediate in its properties. The average oil deposit from this spray was slightly lower than from the others, but the difference, although it may have been a contributing factor, was hardly sufficient to account for all of the difference in scale mortality. Other possible reasons might be differences in the texture of the bark, or more foliage on trees 4 and 7 to hinder the oil from reaching the twig surfaces where the scales were located.

#### DISCUSSION OF RESULTS

From the curves shown in figures 1 and 2 it was found that the average survival for the laboratory sprays containing 0.9 percent of oil ranged from 29 percent at a population density of 5 percent to 90 percent at a population density of 75 percent, and for the spray with 1.5 percent of oil, from 15 to 67 percent at the same population densities; for the 1-percent spray the survival ranged from 21 to 80 percent within the population density limits of 5 to 95 percent, and for the 1.7-percent spray, from 9 to 59 percent within the same limits. In the field sprays, when 2 percent of oil was used, the two oils of 6-percent and 33-percent sulphonatable content killed practically the same proportion of scales, the survival ranging from 25 to 78 percent over the entire population-density range, while for the oil containing 16 percent sulphonatable material the survival was 36 to 96 percent (fig. 3). The oil deposit on the leaves was considerably greater in the field applications, and it appears probable that a larger proportion of the oil was deposited on the foliage relative to that deposited on the twigs, by the field sprays than by the laboratory sprays.

The data given in tables 7 and 8 show no systematic differences in the laboratory tests which could be attributed to the sulphonatable portions of the oils used. In table 8, sprays 5a and 5b, in which the oil used was intermediate in its sulphonatable content, show slightly higher rates of survival than the other sprays in their respective groups. Otherwise the differences in the oil deposit and insect mortality appeared to be entirely random and well within the limits of variation ordinarily encountered in the effects of sprays where a comparatively low mortality is obtained. The results of the field sprays (table 9 and fig. 3) agree with those of the laboratory sprays in failing to show any differences in insecticidal efficiency resulting from differences in the sulphonatable portion of the oils. In all of these tests, therefore, no evidence was found that the sulphonatable portions of the oils had any effect upon the insecticidal action when the other physical and chemical properties of the sprays were uniform.

#### EFFECT OF OILS UPON PLANTS

Little concerning the relation between plant injury and refinement of the oil can be deduced from the laboratory spray tests, since small potted camphor-tree plants with few leaves and a heavy scale infestation may be much more easily injured than trees in the field. Of the



trees to which the field sprays were applied, only the two (nos. 8 and 10) sprayed with the oil containing 33-percent sulphonatable material exhibited any appreciable injury. On February 1 these had lost 75 to 80 percent of their leaves, whereas none of the others had had more than a 10-percent leaf drop. Freezing weather ( $20^{\circ}$  F.) occurred on February 9, and on March 10 tree 10 was completely defoliated and tree 8 had only a few new leaves at the top. However, one of the trees sprayed with the mixed oil and one of the check trees had very few leaves also. The other trees were apparently normal. Other unsprayed trees throughout the city showed great variation in their resistance to the low temperature, and it is not known how much of the injury in the sprayed plot can be ascribed to increased susceptibility resulting from the oil sprays. However, the fact that trees 8 and 10 had by far the greater leaf drop before the freeze may indicate that under normal conditions the less refined oils are more toxic to camphor-trees.

#### SUMMARY

The comparative insecticidal value of three petroleum oils with 6, 16, and 33 percent by volume, respectively, of sulphonatable material was investigated in laboratory and field spraying tests. The oils were applied, in the form of emulsions containing from 0.9 to 2 percent of oil, sodium oleate soap being used as the emulsifier, to camphor-tree plants and trees infested with the camphor scale (*Pseudaonidia duplex* (Ckll.)). The other characteristics of the different oils were substantially the same, so that any detectable differences in scale mortality could be due only to differences in the sulphonatable portions.

When sprays which gave equal oil deposits were compared, the variations in mortality appeared to be entirely random, and no differences were found which could be attributed to the sulphonatable content of the oils.

For making comparisons between the toxicities of different oils, the first condition it was sought to satisfy was that of obtaining equal oil deposits on the plants. Analyses of the oil deposits left by sprays showed that substantially equivalent deposits were obtained when equal concentrations of the different oils were applied. In the laboratory tests the oil deposits produced were independent of the sulphonatable content of the oil but varied directly with the oil content of the spray, ranging from  $3.8 \times 10^{-5}$  cc per square centimeter of leaf surface for a 0.9-percent emulsion to  $6.7 \times 10^{-5}$  cc per square centimeter for a 1.7-percent emulsion. In the field spraying with emulsions containing 2 percent of oil the deposits ranged from  $9.5 \times 10^{-5}$  cc to  $10.1 \times 10^{-5}$  cc per square centimeter of leaf surface.

The average survival for the laboratory sprays containing 0.9 percent of oil ranged from 29 percent at a population density of 5 to 90 percent at a population density of 75 percent, and for the 1.5-percent sprays, from 15 to 67 percent at the same densities; for the 1-percent sprays, from 21 to 80 percent at densities of 5 to 95 percent; and for the 1.7-percent sprays, from 9 to 59 percent within the same density limits. In the field sprays, where 2-percent emulsions were used, the two oils containing 6 and 33 percent of sulphonatable material, respectively, killed the same proportion of scales, the sur-

vival ranging from 25 to 78 percent over the entire population-density range, while for an oil containing 16 percent of sulphonatable material the survival was from 36 to 96 percent.

No conclusion as to the effects of the oils on potted plants was practicable, inasmuch as such results would not be typical of reactions under field conditions; however, in the field tests the trees sprayed with 2-percent emulsions made with oil containing 33 percent of sulphonatable material showed a 75- to 80-percent leaf drop when observed 10 weeks after the spraying date. Trees sprayed with oils of lower sulphonatable content had a maximum leaf drop of less than 10 percent.

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# TRANSMISSION OF SUGARCANE MOSAIC BY THE RUSTY PLUM APHID, *HYSTERONEURA SETARIAE*<sup>1</sup>

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## INTRODUCTION

The spread of mosaic to supposedly resistant varieties of sugarcane such as Co. 281 and C. P. 807 during recent years, and the demonstration of the existence of various strains of the mosaic virus<sup>3</sup> have made evident the need for a detailed study of mosaic spread in Louisiana. This study includes not only the determination of general and specific factors in epidemiology, but a survey for possible additional vectors. Brandes,<sup>4</sup> in 1920, reported experiments showing that sugarcane mosaic was transferred by the corn leaf aphid (*Aphis maidis* Fitch). In this paper Brandes stated that the successful experiments with the corn aphid were of great interest scientifically, but added that it was "not believed that transmission of mosaic is restricted to this insect or to other aphids more abundant on cane." Brandes' findings concerning *A. maidis* were confirmed by other investigators. Despite attempts, by Brandes and later workers, at transmission with other insects, *A. maidis* has until recently remained the sole proved vector of sugarcane mosaic.

In 1930 the senior author began a study of the abundance of *Aphis maidis* in and around sugarcane fields to determine its relationship to the spread of mosaic in Louisiana. At the same time counts were made of other sucking insects found in such locations. Preliminary surveys showed that the rusty plum aphid (*Hysteroneura setariae* (Thomas)) was the most abundant of all aphids found on sugarcane in Louisiana. In view of its abundance and the fact that it had not previously been tried as a vector in the United States, transmission experiments were undertaken. This paper summarizes preliminary experiments showing that this aphid can transmit the mosaic of sugarcane.

## GEOGRAPHIC DISTRIBUTION AND HABITS OF THE RUSTY PLUM APHID

Under the name of the rusty, or rusty-brown, plum aphid, *Hysteroneura setariae* apparently is distributed on plums, with grasses as alternate hosts, throughout most of the United States. Van Dine<sup>5</sup>

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<sup>2</sup> The authors gratefully acknowledge helpful suggestions from P. N. Annand, of the Bureau of Entomology and Plant Quarantine, and R. D. Rands, of the Bureau of Plant Industry.

<sup>3</sup> SUMMERS, E. M. TYPES OF MOSAIC ON SUGAR CANE IN LOUISIANA. *Phytopathology* 24: 1040-1042, illus. 1934.

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<sup>4</sup> BRANDES, E. W. ARTIFICIAL AND INSECT TRANSMISSION OF SUGAR-CANE MOSAIC. *Jour. Agr. Research* 19: 131-138. 1920.

<sup>5</sup> VAN DINE, D. L. A LIST OF THE INSECTS AFFECTING SUGAR CANE IN CUBA. *Trop. Plant Research Found. Bull* 3, 16 pp. 1926.

states that it is generally distributed on sugarcane in Cuba, and Wolcott<sup>6</sup> reports its presence in Puerto Rico. The aphid has been generally found feeding on sugarcane and on other grasses in southern Louisiana. It has also been found feeding on plum twigs in this section, but plum trees are relatively rare around sugarcane fields. The rusty plum aphid feeds on grasses throughout the year in southern Louisiana. Following the low temperatures of the winter of 1934-35, a few individuals were found at all times feeding on perennial *Andropogon* sp. along cane-field ditchbanks.

On sugarcane these aphids are generally found on the collar lobe at the junction of the leaf blade and sheath, but sometimes also in the central whorl or on other parts of the plant. They are usually attended by ants that often form a protective dirt or borer-frass covering over them. They have been found on a large number of grasses that are common in and around cane fields, and some of these are also hosts of sugarcane mosaic. On the larger grasses the aphids are usually found feeding in the same positions as on cane; but on the smaller grasses, such as *Setaria lutescens* and *Eleusine indica*, they have been observed in all positions along the stem and even on the glumes.

The relationship of the rusty plum aphid to mosaic spread apparently has not been studied except by Loftin and Christenson<sup>7</sup> who, working in Cuba, reported the transfer of 10 to 60 *Hysteroneura setariae* from mosaic-infected sugarcane plants to each of 28 healthy plants of the susceptible varieties Cristalina and P. O. J. 2883. No mosaic appeared on any of these 28 plants.

#### EARLY EXPERIMENTAL WORK IN WHICH CAGES WERE USED

Because of very limited greenhouse space, a succession of experiments in small cages or other enclosures was first undertaken in which large numbers of aphids were simply colonized on mosaic-infected plants and left to find their way later to the authentic healthy plants placed alongside in the same cage.

A preliminary experiment was conducted in 1933 in which, under each of 14 lantern globes, one healthy and one infected plant, both of the variety P. O. J. 234, were caged together. Eighty individuals of *Hysteroneura setariae* were released in each of 5 of the globes, 40 *Aphis maidis* in each of 5, and 4 globes were held as checks. One new mosaic-infected plant developed in one of the globes confining *H. setariae*, two in those with *A. maidis*, and none in the checks. This experiment was conducted in an insect-proof greenhouse on ant-proof benches. The plants used were grown in sterile soil, and the healthy plants were from seed pieces that had been observed to be healthy for at least two vegetative generations.

Since this preliminary experiment incriminated *Hysteroneura setariae* as a possible vector, further experiments were instituted. The condensation of moisture and the high temperature in the lantern globes caused a heavy mortality of aphids. Additional experiments were, therefore, conducted in 3- by 4- by 4-foot, insect-proof, cloth cages, having small sliding glass doors through which observations

<sup>6</sup> WOLCOTT, G. N. THE MINOR SUGAR-CANE INSECTS OF PORTO RICO. Jour. Dept. Agr. Porto Rico, v. 5, no. 2, 47 pp., illus. 1921.

<sup>7</sup> LOFTIN, U. C., and CHRISTENSON, L. D. A REPORT ON THE CORN APHIS, APHIS MAIDIS FITCH, IN CUBA. Fourth Cong. Internat. Sugar Cane Technol. Proc. and Bull. 115, 20 pp., illus. 1933.

were made. These cages were placed on ant-proof benches in an insect-proof greenhouse. As in the preceding experiment, the healthy seed cane had a mosaic-free record for at least two vegetative generations, and the plants were grown in sterile soil. Experiments conducted were as follows:

#### EXPERIMENT 1

On December 16, 1933, 18 healthy P. O. J. 234 cane plants were caged with 6 mosaic-infected Louisiana Purple plants. These plants were 4 to 8 inches in height and were all growing in 4-inch paper pots. A total of 750 *Hysteroneura setariae* collected from mosaic-free *Andropogon* sp. were released on the infected plants in the cage. On December 18 it was found that nearly all of the aphids had died as a result of having been parasitized. No mosaic had developed on the 18 plants by January 30, when the experiment was discontinued.

#### EXPERIMENT 2

Six mosaic-infected P. O. J. 36M plants in 5-gallon galvanized-iron cans and 10 healthy Louisiana Purple plants in 4-inch paper pots were caged together. These plants were all from 4 to 8 inches in height. In January 1934, 850 *Hysteroneura setariae*, collected from healthy *Andropogon* sp., were released on the infected plants in the cage. None of the healthy plants had developed mosaic at the time the experiment was discontinued in May 1934.

#### EXPERIMENT 3

Six infected P. O. J. 36M and fifteen healthy Louisiana Purple plants were caged together. On April 19 and 20, 1934, 750 *Hysteroneura setariae*, collected from healthy *Andropogon* sp., were released on the infected plants in the cage. One of the initially healthy plants showed mosaic on June 6, and two additional ones showed well-defined symptoms on July 6. Other healthy cane plants of the same age and source in the greenhouse remained free from the disease.

#### EXPERIMENT 4

Three mosaic-infected and four healthy P. O. J. 234 plants were transplanted together in can no. 1, and three infected and five healthy plants of the same variety were transplanted in can no. 2. These two cans were caged together and 600 *Hysteroneura setariae*, collected from healthy wild grasses, were released in the cage on August 24, 1934. In a second similar cage to be used as a check, three mosaic-infected and five healthy P. O. J. 234 plants were transplanted to can no. 3, and two infected and four healthy plants were transplanted to can no. 4. The healthy and mosaic plants in each of the four cans were separated by an 18-mesh wire screen from the bottom of the cans to about 4 inches above the surface of the soil. The purpose of this separation was to minimize any possibility of mosaic transfer by contact and to prevent mosaic-infected suckers from emerging on the side where the healthy cane was planted. The cans used were of the 5-gallon galvanized-iron type. The plants were about 4 to 8 inches in height when the experiment was started. Three of the initially healthy plants in the aphid-infested cage had developed mosaic by September 30, a fourth by October 6, and a fifth by October 30. No

healthy control plants developed mosaic. Therefore, in this test five out of nine initially healthy plants in the infested cage contracted mosaic.

#### EXPERIMENT 5

Four healthy P. O. J. 36M plants were grown in each of sixteen 5-gallon cans in the greenhouse. On September 25, 1934, when these plants had reached a height of 12 inches, two cans were caged in each of eight insect-proof cloth and glass cages. These cages were 15 by 28 by 29 inches. In five of the cages 25 *Hysteroneura setariae* collected from mosaic-infected plants in the field were placed on each plant by means of a camel's-hair brush. Three cages were held as checks. None of the plants developed mosaic. This possibly may have been due to the large size and lack of vigor in the infested plants.

#### EXPERIMENT 6

One can containing four healthy P. O. J. 234 plants and another containing four infected Co. 281 plants were placed in each of six cages of the same type as used in experiment 5. The plants were from 4 to 10 inches tall. Six hundred *Hysteroneura setariae* were collected from healthy *Andropogon* sp. and released in each of three cages on January 30 and 31 and February 2, 1935. On February 12, 600 *Aphis maidis* collected from *Sorghum halepense* were released in a fourth cage. Cages 5 and 6 were held as checks. All cages were fumigated on March 7 and removed from the plants. At the time the healthy plants were discarded on May 2, no plant had developed mosaic.

It is believed that, in the six experiments listed above, the incidence of mosaic in the infested healthy plants was greatly lessened because the plants were covered by the cages for such long periods that their growth was usually retarded. For this reason they were probably less susceptible to the mosaic virus than succulent, vigorously growing young plants would have been. In all cages in which the aphids had been released, they were observed in the process of feeding to some extent on the sugarcane plants. Information on aphid activity in the cages is limited, owing to the difficulty of making observations in this type of cage. As is the case in the field, most of the rusty plum aphids fed on the collar lobe where the leaf sheath and blade join. It may be that the possibility of transfer by feeding on these tissues, especially in such slow-growing plants, is less than where feeding is done on more succulent tissues such as the leaf spindle. In all seven experiments, 9 of 109 healthy plants infested with *Hysteroneura setariae* from infected plants developed mosaic, and 2 of 9 plants similarly infested with *Aphis maidis* developed symptoms. None of the checks gave evidence of the disease, nor did any mosaic appear in other healthy plants from the same source that were growing in the greenhouse at the same time.

#### EXPERIMENTS IN WHICH GLASS TUBES WERE USED

In an attempt to increase the percentage of transfers by modifications in technique, aphids were confined for 24 hours on mosaic-infected plants in glass tubes one-half inch in diameter and 7 inches in length. The ones remaining alive were then transferred by means of a camel's-hair brush to healthy plants, where they also were confined in glass

tubes. The technique employed consisted of slipping a tube over the growing point of a plant and closing the lower end by packing cotton between the glass and the plant. Aphids were then placed on the plant, and the upper end of the tube was closed with cotton. In most instances the growing point did not extend to the upper end of the tube, and, of course, even the first collar lobe was excluded, and this situation forced the aphids to feed on other parts of the leaves. The aphids used were collected from mosaic-free grass plants in the field. Twenty to thirty aphids were released on each mosaic-infected plant, various susceptible varieties being employed, but the number remaining alive for transfer to healthy plants ranged from 2 to 25, with an average of about 12. Many of the aphids stuck to the sides of the tubes where moisture had condensed, and were incapacitated. The plants used in this series of experiments were from 5 to 6 inches in height and had been grown from cuttings planted in sterile soil in 4-inch clay pots. The mosaic-free cuttings were from commercial canes of susceptible varieties having a history of at least two known healthy vegetative generations. The infected plants upon which the aphids were confined were similar varieties of various known mosaic histories, in many cases the "strain" of virus involved having been previously determined.

During the course of each experiment the healthy plants with their attached tubes were confined in the 18-inch cages, previously described, up to the time when they were fumigated, usually after 6 to 9 days. The experiments were conducted in an insect-proof greenhouse on ant-proof benches. Following fumigation, the tested plants were held for from 60 to 150 days to ascertain definitely whether or not they had contracted the disease. The results of these experiments are given in table 1.

TABLE 1.—*Summary of mosaic-transmission attempts with 2 species of aphids, glass tubes being used for confining the insects on the plants*

Species of aphid	Transfer of aphids		Experiments conducted	Healthy plants used	Plants developing mosaic
	Date	Source			
			Number	Number	Number
<i>Hysteroneura setariae</i> ...	{ Nov. 19, 1934, to May 2, 1935. ....	{ Mosaic sugarcane. ....	15	56	2
	.....	{ Healthy sugarcane. ....	4	12	0
	{ May 5, 1935. ....	{ Mosaic sugarcane. ....	2	6	0
<i>Aphis maidis</i> .....	{ Mar. 1, 1935. ....	{ Mosaic sugarcane. ....	1	3	1
Checks (no aphids) ...	{ Apr. 5 to May 2, 1935. ....	.....	5	24	0
	{ May 5, 1935. ....	.....	1	7	0

<sup>1</sup> Silk bags, ¼ inch in diameter, substituted for glass tubes.

Summarizing the experiments in table 1, it is seen that 2 cases of mosaic developed among a total of 62 plants that had been exposed to presumably viruliferous *Hysteroneura setariae*, and that there was 1 case among 3 plants infested by supposedly viruliferous *Aphis maidis*. The two transfers by *H. setariae* were obtained in an experiment in which 25 aphids were transferred from Louisiana Purple, infected with virus strain 2, on May 2, 1935, to each of 10 plants of healthy P. O. J. 213. The single transfer by *A. maidis* was the result of transferring 14 aphids which had been feeding on Mosaic Co. 281 (strain not determined) on March 1, 1935, to each of three healthy

P. O. J. 234 plants. No evidence of mosaic appeared in the checks in any of these experiments, or in other healthy plants from the same source that were growing in the greenhouse at the same time. No uncaged aphids were ever found in the greenhouse, which was fumigated periodically.

The plants in these experiments were allowed to remain in small pots, where they made poor growth owing to lack of root space and difficulty in getting the proper amount of moisture. This may have been responsible for the small number of mosaic transfers.

#### EXPERIMENTS IN 1935 IN WHICH LARGE CAGES WERE USED

Since it is well known that the expression of mosaic symptoms following infection depends upon further growth and unrolling of new leaves, transmission experiments were undertaken in which the plants employed were kept continuously vigorous. This was accomplished by transplanting them either to flats or beds in the greenhouse and giving them a fairly heavy nitrate fertilization. It was thought that shortening the feeding period of the aphids on the healthy plants to about 8 days and subsequently forcing the plants might increase the percentage of successful transfers with *Hysteronura setariae*. A new series of experiments was, therefore, started in May 1935, in which pedigreed healthy plants growing in 4-inch pots of sterilized soil were transplanted to 14- by 24- by 6-inch flats of sterile soil after 8 days' exposure to viruliferous aphids. The flats were then fertilized and kept under the most favorable growing conditions possible.

In each of these experiments seven mosaic-infected plants were placed in insect-proof glass and cloth cages on ant-proof benches in the insectary. Thirty aphids collected from healthy grasses were placed on each plant with a camel's-hair brush. About 4 hours later eight healthy plants were placed in the cages alternating in position with the infected plants. Twenty-four hours later aphids were transferred from the infected plants to these healthy plants with a camel's-hair brush. To each healthy plant were transferred about 20 aphids which included a few that had in some instances moved from the diseased to the healthy plants prior to the transfers. They were allowed to feed for several days, after which all cages were fumigated. Check plants were given an exactly similar treatment except for the absence of aphids in the cages. Immediately following fumigation the plants with the surrounding soil were removed from the pots and transplanted to flats in the greenhouse, where they remained on ant-proof benches until discarded. Although regular examination were made, no aphids of any kind were ever found in the greenhouse.

As in previous experiments, the plants used were all of varieties known to be susceptible to mosaic. Known strains of the virus were used, whenever available, as a mosaic source, and field-run mosaic cuttings were used at other times. The cages employed in these experiments were 15 by 28 by 49 inches high and 16 by 30 by 33 inches high. All cages used in any given experiment were of the same size.

The results of this series of experiments are shown in table 2. In the transfer occurring in experiment H-1 the aphids had previously fed on Louisiana Purple plants infected with strain no. 1 of the mosaic virus, and in H-5 and H-6 they were from strain no. 2 mosaic infecting



C. P. 28/60. The aphids bringing about the remaining successful transmissions of the disease had fed on P. O. J. 234 infected also with strain no. 2 of the virus. It may be noted that out of 248 plants exposed, a total of 13 transfers of mosaic to originally healthy cane plants was secured in the cages in which *Hysteroneura setariae* was used as the vector. Of 60 healthy plants similarly infested with *Aphis maidis*, 14 developed mosaic. No transfers were observed in 68 check plants or in any plants of similar origin growing in the greenhouse in other experiments.

TABLE 2.—Summary of mosaic-transmission trials with 2 species of aphids in large cages in 1935

Experiment no.	Date of—		Aphid species used	Cages used	Plants used		Transfers of mosaic to healthy plants	Period since first exposure
	Initiation	Termination			Mosaic	Healthy		
				Number	Number	Number	Number	Days
H-1.....	May 18	July 27	<i>Hysteroneura setariae</i> .....	2	12	11	0	23
			<i>Aphis maidis</i> .....	1	3	3	1	
			Check.....	1	3	3	0	
H-2.....	May 22	July 27	<i>H. setariae</i> .....	1	6	7	0	
			Check.....	1	6	5	0	
			<i>H. setariae</i> .....	1	6	3	0	
H-3.....	May 8	July 31	Check.....	1	4	4	0	
			<i>H. setariae</i> .....	4	29	32	0	
			<i>Aphis maidis</i> .....	1	7	8	0	
H-4.....	May 31	Aug 8	Check.....	1	7	8	0	
			<i>H. setariae</i> .....	6	42	38	3	
			<i>Aphis maidis</i> .....	1	7	6	2	
H-5.....	June 14	Sept 2	Check.....	1	7	4	0	15, 27, 39 16, 18
			<i>H. setariae</i> .....	3	9	4	0	
			<i>Aphis maidis</i> .....	2	6	5	1	
H-6.....	June 11	Sept 11	Check.....	2	6	4	0	35
			<i>H. setariae</i> .....	4	28	31	3	
			<i>Aphis maidis</i> .....	1	7	8	0	
H-8.....	July 10	Sept 11	Check.....	1	7	8	0	21, 29, 30
			<i>H. setariae</i> .....	5	35	38	0	
			<i>A. maidis</i> .....	1	7	8	0	
H-9.....	July 12	Sept. 11	Check.....	1	7	8	0	
			<i>H. setariae</i> .....	4	28	30	3	
			<i>A. maidis</i> .....	1	7	6	5	
H-10.....	July 22	Sept. 22	Check.....	1	7	8	0	16, 36, 45 15, 15, 16 21, 21
			<i>H. setariae</i> .....	3	21	24	3	
			<i>A. maidis</i> .....	1	7	8	0	
H-11.....	July 23	Sept. 23	Check.....	1	7	8	0	17, 20, 37 2-17, 3-25
			<i>H. setariae</i> .....	4	28	30	1	
			<i>A. maidis</i> .....	1	7	8	0	
H-12.....	Aug 7	Oct 7	Check.....	1	7	8	0	30
			<i>H. setariae</i> .....	1	7	8	0	

#### MISCELLANEOUS TRANSFER EXPERIMENTS

In August 1935, in an experiment similar to those listed in table 2, one of nine healthy C. P. 28/60 sugarcane plants developed mosaic following the release of 30 *Aphis maidis* per plant from mosaic-infected *Panicum dichotomiflorum*. None of six plants developed mosaic when similarly infested by *Hysteroneura setariae*.

The senior author found the green bug, or spring grain aphid (*Toxoptera graminum* Rond.), feeding on sugarcane in about equal numbers with *Aphis maidis* in some fields in 1934. In March 1935, 25 *T. graminum* collected from *Sorghum halepense* were confined in glass tubes on the whorls of each of three mosaic-infected Co. 281 cane plants for 24 hours. The surviving aphids were then transferred to whorls of three healthy P. O. J. 234 plants, where they were enclosed

in glass tubes for 24 hours and then fumigated. One mosaic transfer occurred, but there were no transfers in the four check plants in this experiment or in similar check plants in other experiments conducted at the same time.

In June 1935 an experiment similar to those listed in table 2 was started in which 30 *Toxoptera graminum* per plant were used as vectors checked against *Aphis maidis*. There was 1 mosaic transfer in 25 healthy C. P. 28/60 plants where *T. graminum* was transferred from mosaic C. P. 28/60, strain no. 2. No mosaic appeared in eight healthy plants to which *A. maidis* was transferred or in eight check plants without aphids. These two transfers suggest that *T. graminum* may be a vector of sugarcane mosaic, but additional experiments are necessary to prove this. This aphid often feeds in the whorls of sugarcane plants in the same manner as does *A. maidis*, for which, owing to its green color, it has no doubt often been mistaken.

Negative results have been obtained from transferring the following insects from mosaic-diseased sugarcane plants to healthy plants in the indicated number of cases: The yellow sugarcane aphid (*Sipha flava* Forbes), 8; the sharp-nosed grain leafhopper (*Draeculacephala mollipes* (Say)), 102; the fulgorid *Sogata furcifera* Horv., 5; and *Haplothrips graminis* Hood, 17.

#### DISCUSSION AND CONCLUSIONS

Results from the experiments reported here show that *Hysteroneura setariae* is a vector of sugarcane mosaic. From table 2 it is calculated that 5.2 percent of the healthy plants developed mosaic when infested with viruliferous *H. setariae*, and 23.3 percent developed mosaic when infested with *Aphis maidis*. These limited data indicate that *A. maidis* is several times more efficient than *H. setariae* as a vector of mosaic.

In all these experiments a higher percentage of transmission might have been obtained with more adequate equipment which would have promoted faster plant growth and consequent expression of symptoms and which would have given more suitable conditions for aphid feeding and multiplication. In spite of improved conditions for the growth of experimental plants reported on in table 2, the incubation periods for both vectors are unusually long. Furthermore, the time required for the appearance of symptoms is seen in the table to have been somewhat longer in most instances for *Hysteroneura setariae* than for *Aphis maidis*. While the differences may have been purely accidental, in view of the small number of direct comparisons, nevertheless a longer incubation period for *H. setariae* might be anticipated since it generally feeds on the collar lobe, which is, of course, less directly connected with the growing point than the young leaf whorl where *A. maidis* feeds.

Proof of mosaic transfer by the rusty plum aphid further complicates the problem of combating mosaic spread in sugarcane. While sugarcane is a natural host for *Aphis maidis* in Louisiana for only a short period in the winter and early in the spring, when other host material is unavailable or scarce, it is one of the preferred hosts of *Hysteroneura setariae* throughout the year. Furthermore, as already mentioned, several grasses bordering cane fields are also acceptable hosts of *H. setariae*. Its role as a vector probably explains several

specific instances of mosaic spread in early summer when frequent inspections showed the entire absence of *A. maidis*, and therefore the abundance of new infections could not be ascribed to this well-known vector.

It seems possible that *Toxoptera graminum* may also prove to be a vector of mosaic, as indicated by two successful transmissions in these experiments. This would be favored by its habit of frequently feeding in the central whorl of the cane plant.

#### SUMMARY

Until 1933 the corn leaf aphid (*Aphis maidis*) had been the only proved vector of sugarcane mosaic, but preliminary experiments in that year showed that the rusty plum aphid (*Hysteroneura setariae*) was also capable of transmitting the disease.

The rusty plum aphid is generally distributed on plums throughout most of the United States, with grasses as alternate hosts. On sugarcane plants it usually feeds at the collar lobe at the junction of the leaf blade and the sheath.

A series of experiments showed that out of 109 healthy plants infested with *Hysteroneura setariae* taken from infected plants, 9 developed the mosaic disease, while 2 out of 9 plants similarly infested with *Aphis maidis* developed symptoms of the disease. A second series of experiments gave 2 transmissions out of 62 exposures with *H. setariae* and 1 out of 3 with *A. maidis*. Another series showed 13 transmissions out of 248 exposures with *H. setariae* and 14 out of 60 with *A. maidis*. Two transfers of the mosaic were made by the feeding of the green bug (*Toxoptera graminum*) after the exposure of 28 healthy plants.

Although not so effective as *Aphis maidis* in transmitting the mosaic, *Hysteroneura setariae* is present and feeding on the cane throughout the year, whereas the former is present only in the winter and early in the spring.



# THE COMPOSITION AND DIGESTIBILITY OF MUNG-BEAN SILAGE, WITH OBSERVATIONS ON THE SILICA-RATIO PROCEDURE FOR STUDYING DIGESTIBILITY<sup>1</sup>

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## INTRODUCTION

Bergeim (3)<sup>2</sup> in 1926 employed a modified procedure for determining the apparent digestibility of food. The method consists of the addition to the food of known amounts of iron oxide or other suitable substance which is eliminated with practical completeness in the feces. The digestibility of any food substance is determined from the ratio of food substance to iron in the food and in the feces. The fecal ratio divided by the food ratio multiplied by 100 gives the percentage of food substance undigested. In practice it is not necessary by this method to measure accurately the total amount of food eaten or feces excreted during an experimental period inasmuch as one is dealing with a change of ratios and not with absolute amounts.

During a study of the digestibility of the proteins of cottonseed (6) Bergeim's method yielded results slightly lower than those obtained by the usual method in which the total amounts of protein consumed and excreted during an experimental period were determined. The lower results were attributed to the incomplete recovery of iron in the feces. By substituting silica for iron oxide and determining the silica ratios in the food and feces, results were obtained which were in close agreement with those obtained by the usual method (7). The experiments were conducted with rats on a diet which contained 1 percent of added silica.

In recent experiments with cows on a ration which contained approximately 3 percent of naturally occurring silica and 0.5 percent of added iron oxide results were obtained which indicated that the silica naturally contained in the feed was superior to iron oxide as a key substance in digestion studies (8). Bergeim (4) reports that Wildt fed sheep wheat straw and used the silica naturally contained therein as an index substance in absorption studies.

Recently the authors had occasion to determine the apparent digestibility of mung-bean silage. Since this feed contained approximately 20 percent of silica (dry-matter basis) or more than six times the amount present in rations previously employed (8) it appeared of interest to make the digestion studies by both the silica-index procedure and the usual method. It is the purpose of the present paper to report the results of these studies and to point out likely sources of error in the silica-ratio procedure.

Only one analysis of mung-bean silage is reported by Henry and Morrison (9), and so far as the authors are aware, no determinations have been made of its digestibility.

<sup>1</sup> Received for publication Nov. 5, 1935; issued July 1936.

<sup>2</sup> Reference is made by number (italic) to Literature Cited, p. 894.

## MATERIAL AND METHODS

The silage was made from Golden mung beans, a tall variety which produces few seed pods. The plants were cut and ensiled before they had fully matured, while the bean pods were still green. The silage when taken from the silo had a characteristic but not unpleasant odor and in this respect differed from silage made of many of the legumes. On drying, it developed a pungent odor.

Weekly samples of silage were taken from the silo for several months previous to the digestion trial, and these were combined to form three composite samples representing material from the upper, middle, and lower parts of the silo. The samples were dried and reserved for analysis.

Nine 1-pound samples were taken from the lower part of the silo for silica and proximate analysis at the time the digestion trial was in progress.

The experimental animals were four milk cows, three of which had received mung-bean silage in their ration for several months previous to the digestion trial. They were stanchioned in the barn during the entire trial except for about 30 minutes each day, when they were exercised in a dry lot in the presence of a caretaker. The digestion trial was of 17 days' duration, 10 days' preliminary and 7 days' experimental. During the first part of the preliminary period the maximum amount of silage which the cows would consume was determined. During the 7-day experimental period, the silage was fed four times daily in four equal portions. In this way uneaten residues were avoided.

During the experimental 7-day period feces collections were made by hand. The total amount of feces voided daily was weighed and a one-twentieth aliquot taken for analysis and dry-matter determinations. A tenth of the one-twentieth aliquot was preserved in acid alcohol for nitrogen determinations. To obtain data on the constancy of the silica output, a small sample of each defecation from each cow was taken during a 24-hour period. All analyses of feed and feces were made according to methods adopted by the Association of Official Agricultural Chemists (1).

## RESULTS

The average composition of mung-bean silage calculated from the analysis of three composite samples was found to be as follows: Moisture, 67.33 percent; crude protein, 4.81; crude fiber, 7.26; fat, 1.38; nitrogen-free extract, 13.12; and ash, 6.10 percent.

The percentage composition of mung-bean silage used in the digestion trial and the percentage composition of the feces voided by cows receiving the silage are given in table 1. The figures given in this table were used in calculating the digestibility coefficients presented in table 2.

The composition of mung-bean silage as given in table 1 differs appreciably from that reported by Henry and Morrison (9) in the percentage of ash and crude fiber, being higher in ash and lower in crude fiber. These authors report the ash and crude-fiber content of silage made from "nearly mature" mung beans as 1.2 and 9.8 percent, respectively. This difference between the two silages may be attributed in

part to varietal differences in composition of the plants and possibly to the manner in which they were handled before ensiling. Unless the plants are cut before the pods begin to turn brown many of the leaves and pods shatter when handled. The loss of leaves tends to decrease the percentage of ash and increase the percentage of fiber in the hay.

Foreign material may have contributed to the high ash content of the silage used in the present study since the plants were grown during a dry, dusty season. Approximately 75 percent of the ash in the silage was silica. Silica constituted only about 45 percent of the ash in the composite samples of silage. Similarly about 50 percent of the ash of wheat straw and 30 percent of the ash of timothy hay is silica. Analyses of soybean silage compiled by Becker et al. (2) show the ash content of this material to vary between 8.71 and 19 percent of the dry weight.

TABLE 1.—*Composition of mung-bean silage and of feces voided by cows which received the silage during a 7-day digestion trial*

Material analyzed and cow no	Moisture	Crude protein	Crude fiber	Ether extract	Nitrogen-free extract	Ash
	<i>Percent</i>	<i>Percent</i>	<i>Percent</i>	<i>Percent</i>	<i>Percent</i>	<i>Percent</i>
Original silage.....	72.67	3.79	5.21	1.24	9.86	7.23
Dry silage.....		13.88	19.07	4.56	36.04	26.45
Cow 1. Dry feces.....		11.98	17.77	2.23	25.89	42.13
Cow 2. Dry feces.....		11.29	17.70	2.22	26.84	41.95
Cow 3. Dry feces.....		12.08	18.04	2.26	28.47	39.15
Cow 4. Dry feces.....		11.13	19.02	2.41	29.64	37.80

TABLE 2.—*Dry matter and nutrients ingested, voided, and digested during a 7-day period by cows on mung-bean silage*

Cow no and items compared	Dry matter	Protein	Ether extract	Crude fiber	Nitrogen-free extract	Ash
Cow 1:						
Consumed.....kilogram.....	46.8602	6.5042	2.1368	8.9362	16.8884	12.3945
Voided.....do.....	25.4079	3.0439	.5666	4.5150	6.5781	10.7043
Digested.....do.....	21.4523	3.4603	1.5702	4.4212	10.3103	1.6902
Do.....percent.....	45.7	53.2	73.4	49.4	61.0	13.6
Cow 2:						
Consumed.....kilogram.....	52.0672	7.2269	2.3743	9.9292	18.7650	13.7717
Voided.....do.....	27.7243	3.1301	.6155	4.9072	7.4415	11.6303
Digested.....do.....	24.3429	4.0968	1.7588	5.0220	11.3235	2.1414
Do.....percent.....	46.7	56.6	74.0	50.5	60.3	15.5
Cow 3:						
Consumed.....kilogram.....	31.2401	4.3361	1.4245	5.9575	11.2589	8.2630
Voided.....do.....	16.4404	1.9860	.3710	2.9653	4.6806	0.4364
Digested.....do.....	14.7997	2.3501	1.0529	2.9917	6.5783	1.8266
Do.....percent.....	47.3	54.2	73.9	50.2	58.4	22.1
Cow 4:						
Consumed.....kilogram.....	31.2401	4.3361	1.4245	5.9575	11.2589	8.2630
Voided.....do.....	18.4581	2.0544	.4448	3.5107	5.4710	6.9772
Digested.....do.....	12.7820	2.2817	.9797	2.4468	5.7879	1.2858
Do.....percent.....	40.9	52.6	68.7	41.0	51.4	15.5
Average, digested.....do.....	45.2	54.2	72.6	47.8	57.8	16.7

## DIGESTIBILITY DETERMINED BY USUAL METHOD

Table 2 contains data on the dry matter and nutrients eaten, voided, and digested by cows receiving mung-bean silage. With but few exceptions, the digestibility figures obtained with the individual cows are in close agreement. Cow 4 gave the lowest values for all constituents except ash.

A comparison of the digestibility coefficients presented in table 2 with those reported for many other silages shows that the digestibility of the dry matter in mung-bean silage is low, being 45.2 percent. The average digestibility of dry matter in corn, cowpea, kafir, soybean, and vetch silage varies from 55 to 69 percent (9). The digestibility of the fat is relatively high. The digestibility of the protein, 54.2 percent, is about the same as that of corn and cowpea silages but less than that of soybean silage. The digestibility of the crude fiber, 47.8 percent, is less than that of corn silage and about the same as that reported for cowpea and soybean silages.

From the results given in table 2, the pounds of digestible nutrients in 100 pounds of fresh mung-bean silage containing 72.7 percent of moisture have been calculated to be 2 pounds of protein, 8.2 pounds of carbohydrates, and 0.9 pounds of fat.

Data on breed, age, average weight, and feed consumption of the cows are given in table 3.

TABLE 3.—Age, breed, weight, and feed consumption of cows used in digestion trial

Cow no.	Age		Breed	Average weight <sup>1</sup>	Fresh silage consumed daily	Total digestible nutrients consumed daily
	Years	Months				
1.....	5	7	Ayrshire.....	Pounds 1,157	Pounds 54	Pounds 6.64
2.....	5	4	Guernsey.....	885	60	7.38
3.....	6	2	Jersey.....	717	36	4.43
4.....	4	0	do.....	775	36	4.43

<sup>1</sup> Average of 8 weights taken during the digestion trial

## DIGESTIBILITY DETERMINED BY SILICA-RATIO PROCEDURE

A uniform distribution of silica in the feed is essential for the determination of the apparent digestibility of a food substance by the silica-ratio procedure. It is likewise essential that the silica follow the food through the intestinal tract without stratification and that it be eliminated in like proportion in all parts of the feces. If this is the case, random samples of feces may be taken without making total collections.

Separate samples of the silage taken at the time the digestion trial was in progress showed considerable variation in silica content. On a dry-matter basis, the minimum silica content of nine 1-pound samples was 18.34 percent; the maximum, 22.54 percent; and the average, 19.91 percent. The percentage of silica was unrelated to the percentage of other constituents except ash, and no regular variation was observed in the silica content of samples taken on consecutive days. The variations that did occur and that were considered significant resulted in all probability from the uneven



distribution of silica in the silage. They represent sources of error in the procedure in that the food substance to silica ratio of the silage is not constant from day to day. This error was not encountered in previous experiments in which silica was present in smaller amounts and was well distributed in the feed (8).

The silica content of separate collections of feces from a single animal during a 24-hour period showed even wider variations. For example, the minimum silica content, the maximum, and the average of feces voided by cow no. 1 during the digestion period were 20.36, 29.61, and 26.20 percent, respectively, on a dry-matter basis. Variations of the same order were observed in the 24-hour samples of feces from the other three cows. Since with the exception of ash, the percentage of food constituents in the feces remained fairly constant during the 24-hour period, these variations in silica content produced variations of equal magnitude in the food substance silica ratios. Digestibility coefficients calculated from these ratios therefore showed no agreement, and it became evident that under these experimental conditions random sampling of feces was not good practice.

Since composite samples of the total amount of feces voided by each cow during the 7-day experimental period were available, silica determinations were made on these and digestibility coefficients obtained which were slightly lower than those obtained by the usual method. Thus the digestibility of the protein calculated from the silica ratio was 48.06 percent for cow 1; 50.65 percent for cow 2; 45.34 percent for cow 3; and 45.34 percent for cow 4. In these calculations the average silica content of the dry silage, 19.91 percent, was used.

Consistently low digestibility coefficients calculated by this method indicate an incomplete recovery of silica from the feces and suggest an appreciable metabolism and subsequent elimination of silica through other channels. Forbes (5) observed a retention of silicon by milking cows and suggested that storage took place through the growth of hair. He also observed an excretion of small amounts of silica in the urine but none in the milk.

In the present experiment over 85 percent of the calculated amount of silica ingested was recovered in the feces. This apparent metabolism of natural-occurring silica contributes a serious error to the method of study.

That under the conditions of this experiment the silica-ratio procedure for studying digestibility had no advantage over the older method of procedure is obvious. That under more favorable conditions the method is capable of yielding accurate results has been demonstrated in previous experiments (7, 8).

#### SUMMARY

The apparent digestibility of mung-bean silage is reported for the first time. The digestion experiments were carried out with four milk cows receiving a ration composed solely of mung-bean silage. The fresh silage contained 3.79 percent of crude protein, 5.21 of crude fiber, 1.24 of fat, and 9.86 of nitrogen-free extract. The average digestibility coefficients were 45.2 for dry matter, 54.2 for protein, 47.8 for crude fiber, 72.6 for fat, and 57.8 for nitrogen-free extract.

It was found that the short method for studying digestibility which makes use of the change in ratio of food substance to silica in the feed as it passes through the intestinal tract was not applicable under the conditions of this experiment. The food substance to silica ratio of the feed varied from day to day. The same ratio in the feces showed wide variations during a 24-hour period, making the random sampling of feces impossible. The cause of these irregularities was not determined but was presumed to be due to (1) an uneven distribution of silica in the silage and (2) stratification and loss of silica in its passage through the intestinal tract.

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## INHERITANCE OF RESISTANCE TO THE COMMON MOSAIC VIRUS IN THE BEAN<sup>1</sup>

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### INTRODUCTION

The mosaic virus of the common bean (*Phaseolus vulgaris* L.) is coextensive with bean culture in the United States and Canada. Reports of the disease in other countries appear in the literature, and the work of Rands and Brotherton (19)<sup>3</sup> with varieties of beans from all parts of the world suggests that the virus is cosmopolitan in its distribution.

Owing to the fact that disease-free seed is difficult to produce and that elimination of the virus from infected seed is not possible, the only means of control available at present is the use of resistant or immune varieties. Michigan Robust is the only commercial variety of field beans that is immune. However, immune selections of the Great Northern variety of field bean (17) and the Stringless Green Refugee canning bean (18) are being released by the Idaho Agricultural Experiment Station. Two new mosaic-resistant snap beans of the Stringless Refugee type have been released under the designations of U. S. No. 1 and U. S. No. 5 by the United States Department of Agriculture since this investigation was started. Corbett Refugee, a selection from commercial Stringless Green Refugee, was the only immune canning-type bean available for the present investigation.

Several investigators have classified bean varieties according to their degree of resistance. Pierce and Walker, as reported by Clark (3), for example, group the varieties tested into three classes. Class 1 includes the varieties (Stringless Green Refugee, Refugee Wax, Red Valentine, etc.) that are susceptible to the common or Refugee-type virus and develop pronounced symptoms when infected; in class 2 are placed the tolerant varieties (Improved Kidney Wax, Full Measure, Giant Stringless, etc.), which are readily infected but which ordinarily do not develop marked symptoms; and in class 3 are grouped the resistant or immune varieties. In this paper are reported the results of crossing bean varieties belonging to the extremes (classes 1 and 3) in the above grouping in order to determine the inheritance of resistance to the common bean mosaic virus. In the early stages of the breeding work it was observed that resistance did not conform to any simple law of inheritance, and for that reason the program of hybridization was limited to a few varieties with a view to studying them in considerable detail.

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<sup>2</sup> The writer wishes to express his appreciation for the helpful assistance given during the course of this investigation by Dr. E. A. Brink and for the advice given by Dr. J. C. Walker, of the College of Agriculture, University of Wisconsin.

<sup>3</sup> Reference is made by number (italic) to Literature Cited, p. 914.

## REVIEW OF LITERATURE

Since Iwanowski (7), in 1899, first mentioned the occurrence of a virus disease on beans, numerous observations have been published dealing largely with symptoms, environmental relations, transmission, distribution, and economic losses caused by the disease. Only one paper deals to any extent with the subject with which we are concerned, namely, the inheritance of resistance to bean mosaic.

McRostie (9), in 1921, reported the results of crossing resistant Michigan Robust with Flat Marrow and with a strain designated as Selection B. According to his data, McRostie obtained a high degree of resistance in the  $F_1$  generation in certain cases. If one may assume that the first variety he lists in each case is the maternal parent, then it would appear that in crosses between resistant and susceptible varieties the  $F_1$  was more resistant when the resistant variety was used as the female parent than as the male parent. The following is a summary of table 3 of McRostie's paper, showing the reactions of a series of  $F_1$  individuals:

Robust  $\times$  Flat Marrow, *b, b, b, b, b, b*.<sup>4</sup>  
 Flat Marrow  $\times$  Robust, *e, d, d, d, b, b*.  
 Robust  $\times$  Selection B, *d, d*.  
 Selection B  $\times$  Robust, *b, d*.  
 Well's Red Kidney  $\times$  Robust, *b*.

The  $F_1$  plants from the Robust  $\times$  Flat Marrow cross, as listed in McRostie's table, were all highly resistant (class *b*), while four out of six of the plants from the reciprocal cross were more or less severely infected. McRostie makes no mention of the apparent difference in the reciprocal crosses. However, this difference is not evident in the  $F_1$  generation of Robust  $\times$  Selection B and reciprocal. Both plants from the cross Robust  $\times$  Selection B were heavily infected. One plant was highly resistant and one very susceptible in the cross Selection B  $\times$  Robust. McRostie indicates that the results show susceptibility to be dominant, or partially so, over resistance. In the  $F_2$  generation a ratio approximating nine susceptible to seven resistant was obtained when all  $F_2$  results were grouped together without reference to the varieties used or the direction in which the crosses were made. Variations in degree of resistance and susceptibility in the  $F_2$  from different crosses are suggested in the  $F_2$  table, but McRostie attributes these to the small numbers involved. He states (9, p. 22):

In view of the ratio reported, we must assume that two factors are concerned in producing susceptibility or resistance to this disease. The presence of both of these factors in the dominant condition goes to make susceptibility. The presence, on the other hand, of one or both of their recessive allelomorphs in the homozygous condition tends to produce a plant that is resistant to the disease in question.

McRostie was handicapped by the lack of a technique that would insure infection of all susceptible plants. In the greenhouse he used a rubbing method of inoculation, but in the field natural dissemination of the virus was relied upon, every third row being planted with seed from diseased plants.

Pierce and Walker (18), crossing Corbett Refugee and Stringless Green Refugee, found the  $F_1$  hybrids to be resistant to mosaic and succeeded in isolating resistant progenies from resistant  $F_2$  plants.

<sup>4</sup> The letters *a* to *e* indicate varying degrees of infection, *a* indicating no infection and *e* severe infection.

In the two investigations just reviewed two different ideas are expressed. McRostie reported resistance to be recessive, while Pierce and Walker found resistance to be dominant to susceptibility.

#### METHODS AND MATERIALS

The Stringless Green Refugee variety, a prolific green snap bean of high quality, which is extensively grown for canning, was used as the susceptible parent throughout the present investigation. It is very susceptible to mosaic, and the disease is an important agent in reducing the yields. Michigan Robust (Michigan No. 40520), a white pea bean, was used as a resistant parent. This strain is immune to the mosaic virus employed in these studies. Corbett Refugee, an immune selection from the Stringless Green Refugee variety, was also used as a resistant parent.<sup>5</sup>

Hybridizations between disease-free parent plants were made in the greenhouse, care being taken to avoid self or otherwise uncontrolled pollination. The flowers were emasculated when the edges of the standard petal of the corolla were just beginning to separate. Buds manipulated earlier were usually shed, and buds almost fully open were found to be self-pollinated.

The flowers were pollinated immediately after emasculation, tagged, and wrapped in paper or cotton to guard against foreign pollen and to prevent destructive drying of the exposed floral parts. The immunity of the resistant parent lines was established by inoculating representative samples with the mosaic virus. A few flowers of each plant used as a parent in crosses were self-pollinated and the reaction of the offspring to the virus was determined. All descendants from "selfed" resistant parents were immune. Likewise, all plants produced from self-pollinated flowers of susceptible parents were susceptible. This procedure gives conclusive evidence that plants used as resistant parents were immune and upon "selfing" transmitted the immunity to all of their offspring. It also proves that plants used as susceptible parents were "true" susceptibles.

The hybrid offspring and the parent strains were grown in a cheesecloth cage out of doors. With this method no difficulty was experienced in keeping the plants healthy. The general procedure in handling hybrid plants was as follows: A few  $F_1$  individuals were inoculated with the virus in order to obtain an index of their reaction to the disease. The rest of the  $F_1$  plants were kept healthy by growing them in the cheesecloth cage mentioned above;  $F_2$  seed harvested from these plants, therefore, was disease-free. The  $F_2$  plants were first exposed to the virus as seedlings. These inoculated plants were selfed and harvested separately. The  $F_3$  generation, therefore, was derived from  $F_2$  individuals whose reaction to the virus had been established. There was tested also an  $F_3$  generation from plants that had been kept disease-free in the  $F_1$  and  $F_2$ .

A portion of the seed from  $F_2$  plants classified as resistant was planted and observed in the greenhouse in order to establish the fact that these plants were actually immune and were not carrying the virus in a masked condition. No diseased plants occurred among the offspring of such tested plants.

<sup>5</sup> Seed of the Corbett Refugee strain and disease-free seed of the Stringless Green Refugee variety were obtained from W. J. Zaunmeyer, Division of Fruit and Vegetable Crops and Diseases, Bureau of Plant Industry, U. S. Department of Agriculture.

Owing to the "hard-shell" character of the hybrid seed it was found necessary to scarify all seeds before planting, in order to obtain uniform germination. Newly harvested seed, if scarified, will germinate immediately upon being planted.

A leaf-rubbing method of inoculation was used, which consisted of macerating leaves from diseased plants in a mortar and expressing the juice through cheesecloth. The fully expanded primary leaves of the plants to be inoculated were rubbed on the upper surface along the midrib with a small piece of cheesecloth dipped in the infective juice. A small quantity of quartz sand was added as an abrasive agent. Fajardo (6) reported from 80- to 100-percent infection with this method. During the course of the present investigation no difficulty was encountered in producing 100-percent infection in plants known to be susceptible if a second inoculation, into the first compound leaf, was given a few days after the first. Figure 1 is a photograph



FIGURE 1—F<sub>2</sub> bean plants 30 days after inoculation: A, Susceptible plant showing mosaic symptoms; B, resistant plant. The primary leaves show inoculation injury.

showing (A) susceptible and (B) resistant plants 30 days after inoculation. Evidence of the inoculation injury may be seen on the primary leaves.

The virus inoculum was obtained from the descendants of a single plant of the Stringless Green Refugee variety grown in the United States Department of Agriculture bean trials at Greeley, Colo., in 1931. The plant exhibited mosaic symptoms similar to other diseased plants in the vicinity and was selected as representative of the ordinary or common type of bean mosaic. It is the writer's opinion that this virus is identical with bean virus no. 1 as reported by Pierce (16). Disease-free Refugee plants were inoculated with virus extract from diseased offspring of this plant. The culture has been maintained by repeated inoculations of disease-free seedlings. This material has been the sole source of infective tissue for testing the reaction of parents and hybrid progenies to the disease. The plan was adopted

to avoid any complication that might result if different strains of the bean mosaic virus exist.

Disease-free seedlings of the Stringless Green Refugee variety were used as checks throughout the investigation. The infectivity of each extracted sample of inoculum used was established by observing the symptoms produced on plants of this stock. Every fifth row in greenhouse plantings was a check. In the field the check rows, containing approximately 30 plants each, were located at random. All susceptible check plants became infected following inoculation. This should be sufficient check on the technique employed and upon the environmental conditions prevailing to establish with certainty the fact that all susceptible plants in the segregating progenies were infected with the virus. The checks were included in all plantings, and in each case 100-percent infection was obtained by means of two inoculations as mentioned above.

Michigan Robust and Corbett Refugee were used as resistant checks. In no case did resistant check plants become infected. The beans were planted in the genetics plot on the University of Wisconsin campus at Madison, Wis., and were more or less isolated from other beans each year.

## RESULTS

### BEHAVIOR OF F<sub>1</sub> GENERATION OF MICHIGAN ROBUST × STRINGLESS GREEN REFUGEE AND RECIPROCAL

In the F<sub>1</sub> generation of the Michigan Robust × Stringless Green Refugee combinations the reciprocal crosses were markedly unlike. The results of inoculating F<sub>1</sub> individuals of the cross susceptible ♀ × resistant ♂<sup>6</sup> are presented in table 1.

TABLE 1.—Reaction of F<sub>1</sub> generation of susceptible (*Stringless Green Refugee*) ♀ × resistant (*Michigan Robust*) ♂ and reciprocal to common bean mosaic

#### STRINGLESS GREEN REFUGEE × MICHIGAN ROBUST

Parent no.	Plant no	F <sub>1</sub> plants			Parent no.	Plant no	F <sub>1</sub> plants		
		Tested	Healthy	Dis-eased			Tested	Healthy	Dis-eased
		Num-ber	Number	Num-ber			Num-ber	Number	Num-ber
1-2×1-16.....	34-3.....	1	0	1	3-1×3-31.....	101.....	1	0	1
1-2×1-17.....	35-2.....	1	0	1	3-2×3-21.....	102.....	3	0	3
1-2×1-22.....	36-4.....	1	0	1	3-3×3-20.....	103.....	2	0	2
1-3×1-11.....	37-3.....	1	0	1	3-3×3-23.....	104.....	1	0	1
1-3×1-12.....	38-3.....	1	0	1	3-3×3-24.....	105.....	2	0	2
1-8×1-12.....	39-3.....	1	0	1	3-4×3-26.....	106.....	2	0	2
1-10×1-12.....	40-5.....	1	0	1	Total.....		21	0	21
1-10×1-15.....	41-3.....	1	0	1					
3-1×3-20.....	100.....	2	0	2					

#### MICHIGAN ROBUST × STRINGLESS GREEN REFUGEE

1-11×1-8.....	44-5.....	1	1	0	3-21×3-4.....	107.....	1	1	0
1-16×1-2.....	45-2, 3.....	2	1	1	3-22×3-1.....	108.....	2	1	1
1-20×1-99.....	46-5.....	1	1	0	3-24×3-3.....	109.....	2	2	0
1-23×1-96.....	47-4.....	1	1	0	3-27×3-2.....	110.....	1	1	0
1-17×1-2.....	49-1, 2.....	2	1	1	Total.....		17	14	3
1-20×1-88.....	50-1, 2.....	2	2	0					
1-22×1-2.....	51-1, 2.....	2	2	0					

<sup>6</sup> To avoid confusion in presenting and discussing these results, the maternal parent has been listed first in each case.

Twenty-one plants were tested, all of which proved to be susceptible. The results of inoculating  $F_1$  individuals from the reciprocal cross are given in table 1. Only 3 out of 17 plants tested were susceptible to mosaic. These results might suggest that the  $F_1$  plants were not hybrids but accidental selfs, since their reactions to the mosaic virus are similar to the reactions of the respective female parents. The Michigan Robust variety has white seeds, white flowers, and an indeterminate type of growth, whereas Stringless Green Refugee has purple-mottled seeds, pink blossoms, and a determinate type of growth. It was possible, therefore, to establish definitely the hybridity of the  $F_1$  plants by the characters shown in this generation and by segregation in the  $F_2$  generation.

The different behavior of reciprocal hybrids in relation to the mosaic virus must be explained in some other manner. The  $F_1$  plants tend strongly to resemble the maternal parent with respect to susceptibility. If the female parent is susceptible to mosaic the offspring are susceptible, and if a resistant individual is used as a mother plant most of the offspring are resistant. These results are contrary to the Mendelian type of inheritance, where except in cases of sex linkage both parents contribute equally to the offspring.

In considering the additional data given below, a brief account of the writer's (15) results on the inheritance of a variegated leaf character in the bean will be helpful. Hybrids were made between plants with variegated leaves and normal green plants. All  $F_1$  plants from the variegated ♀ × green ♂ cross were variegated. Seven out of eight  $F_1$  plants from the reciprocal cross (normal green ♀ × variegated ♂) were normal green; one plant had a slight variegation on one leaf. This difference in reciprocals was again evident in the  $F_2$  generation. In the second generation of variegated ♀ × green ♂, 84 percent variegated and 16 percent green plants were obtained. In the reciprocal cross (green ♀ × variegated ♂) 93 percent green and 7 percent variegated plants were found. These results suggest that the character of the  $F_1$  and  $F_2$  offspring is largely governed by the maternal parent and that the variegated condition is governed by the cytoplasm. The occurrence of some variegated offspring when the variegated plants are used as paternal parents suggests that the causal agency is introduced, to some extent, with the pollen tube at the time of fertilization.

Further discussion and explanation of the variegated character and its relation to mosaic resistance will be found in the section entitled "Discussion."

#### BEHAVIOR OF $F_2$ GENERATION OF MICHIGAN ROBUST × STRINGLESS GREEN REFUGEE AND RECIPROCAL

The  $F_2$  individuals were obtained from plants that were not inoculated in the  $F_1$  generation. Recent workers have reported the transmission of mosaic by the pollen from diseased plants. Reddick (20) first reported this for beans in 1931. Nelson and Down (10) concluded that one-fourth of the ovules and approximately one-fourth of the pollen grains of diseased plants are infected and are instrumental in transmitting the disease to the next generation. Since it was desirable to avoid any complications that might arise from infection of some of the young embryos borne on susceptible mother plants or from infection of ovules of resistant mother plants



by diseased pollen from susceptible individuals, the plants that gave rise to the  $F_2$  progenies were grown in a cheesecloth cage to keep them disease-free.

The striking difference in behavior of the reciprocal crosses shown in the  $F_1$  is again apparent in the  $F_2$  generation. The  $F_2$  results of the susceptible  $\times$  resistant cross are shown in table 2. Out of a total of 4,728 plants inoculated with the mosaic virus, only 60 plants, or 1.27 percent, remained healthy. On the other hand, 4,668 plants, or 98.73 percent, became infected.

TABLE 2.—Reaction of  $F_2$  generation of susceptible (*Stringless Green Refugee*)  $\varnothing \times$  resistant (*Michigan Robust*)  $\sigma$  and reciprocal to common bean mosaic

STRINGLESS GREEN REFUGEE  $\times$  MICHIGAN ROBUST

Progeny no	F <sub>2</sub> plants					Progeny no	F <sub>2</sub> plants				
	Number			Percent			Number			Percent	
	Tested	Healthy	Diseased	Healthy	Diseased		Tested	Healthy	Diseased	Healthy	Diseased
34-1.....	321	1	320	0 31	99 69	39-1.....	464	3	461	0 65	99 35
34-2.....	307	2	305	.65	99 35	39-2.....	497	5	492	1.01	98 99
35-1.....	426	6	420	1.41	98.59	40-1.....	41	0	41	.00	100.00
36-1.....	253	3	250	1 19	98 81	40-2.....	130	0	130	.00	100 00
36-2.....	212	8	204	3.77	96 23	40-3.....	403	1	402	.25	99 75
36-3.....	232	6	226	2.59	97 41	40-4.....	27	0	27	.00	100 00
37-1.....	268	3	265	1.12	98 88	41-1.....	221	6	215	2 71	97 29
37-2.....	284	6	278	2.11	97.89	41-2.....	294	7	287	2 38	97.62
38-1.....	218	0	218	.00	100 00						
38-2.....	130	3	127	2 31	97 69	Total	4,728	60	4,668	1 27	98.73

MICHIGAN ROBUST  $\times$  STRINGLESS GREEN REFUGEE

44-1.....	324	178	146	54 94	45 06	46-3.....	27	23	4	85.19	14.81
44-2.....	148	86	62	58.11	41.89	46-4.....	68	43	25	63.24	36.76
44-3.....	223	117	106	52.47	47.53	47-1.....	59	37	22	62 71	37.29
44-4.....	419	219	200	52.27	47 73	47-2.....	57	38	19	66 67	33 33
45-1.....	367	188	179	51.23	48 77	47-3.....	162	99	63	61.11	38.89
46-1.....	27	18	9	66 67	33 33						
46-2.....	25	15	10	60.00	40 00	Total	1,906	1,061	845	55 67	44 33

The  $F_2$  results from the reciprocal cross (resistant  $\times$  susceptible) are presented in table 2. Of the 1,906 plants tested, 845 became diseased, and 1,061 remained healthy. This is a ratio of 55.67 percent resistant to 44.33 percent susceptible.

The results obtained in the second generation, though not so striking as the  $F_1$  results, still show a preponderating influence of the grandmaternal parent. In the cross susceptible  $\times$  resistant more than 90 percent of the plants were susceptible in every case. Likewise, in the cross resistant  $\times$  susceptible more than 50 percent of the plants were resistant in every case. The result of 55.67 percent resistant and 44.33 percent susceptible when the cross is made this way (resistant  $\times$  susceptible) becomes more significant if it is realized that in no family was there more than 48.77 percent susceptibility or less than 51.23 percent resistance. It should be noted that certain  $F_1$  plants and  $F_2$  progenies are identical reciprocals, i. e., from crosses between the same parent plants. For example, the  $F_1$  plant 34-3 (table 1) is from a cross between parent 1-2 (*Stringless Green Refugee*) as female and parent 1-16 (*Michigan Robust*) as male. The  $F_1$  individuals 45-2 and 45-3 (table 1) are from the reciprocal cross, parent 1-16 (*Michigan Robust*)  $\times$  parent 1-2 (*Stringless Green Refugee*).

BEHAVIOR OF F<sub>3</sub> GENERATION OF MICHIGAN ROBUST × STRINGLESS GREEN  
REFUGEE AND RECIPROCAL

The F<sub>3</sub> plants tested were descendants of self-pollinated F<sub>2</sub> individuals whose reaction to the virus had been determined in the second generation. The results of inoculating F<sub>3</sub> progenies have been grouped in tables 3 to 6, according to the original cross and the reaction of the F<sub>2</sub> parent.

TABLE 3—Reaction to common bean mosaic of F<sub>3</sub> generation of susceptible (*Stringless Green Refugee*) ♀ × resistant (*Michigan Robust*) ♂ and reciprocal, derived from plants resistant in F<sub>2</sub>

STRINGLESS GREEN REFUGEE × MICHIGAN ROBUST

Progeny no.	F <sub>3</sub> plants					Progeny no.	F <sub>3</sub> plants				
	Number			Percent			Number			Percent	
	Tested	Healthy	Dis- eased	Healthy	Dis- eased		Tested	Healthy	Dis- eased	Healthy	Dis- eased
35-1-1...	32	21	11	65 63	34 37	39-1-1...	205	31	174	15 12	84 88
35-1-2...	147	81	66	55 10	44 90	39-2-1...	11	6	5	54.55	45.45
36-1-1...	33	30	3	90 91	9 09	39-2-2...	12	5	7	41.67	58.33
36-1-2...	50	50	0	100 00	.00	39-2-3...	14	12	2	85 71	14 29
36-1-3...	242	240	2	99 17	.83	40-3-1...	12	10	2	83 33	16 67
36-1-4...	28	27	1	96.43	3.57	41-1-1...	18	18	0	100.00	.00
36-1-5...	11	11	0	100 00	.00	41-1-2...	86	74	12	86 05	13 95
36-2-1...	356	348	8	97 75	2.25	41-1-3...	176	170	6	96.59	3 41
36-2-2...	132	111	21	84 09	15 91	41-1-4...	90	74	16	82 22	17 78
36-2-3...	244	186	58	76 23	23 77	41-1-5...	161	156	5	96.89	3 11
36-2-4...	26	17	9	65 38	34 62	41-1-6...	77	76	1	98 70	1 30
36-2-5...	10	4	6	40.00	60 00	41-1-7...	16	16	0	100 00	.00
36-3-1...	135	117	18	86 67	13 33	41-1-8...	18	17	1	94 44	5 56
36-3-2...	201	97	104	48.26	51.74	41-2-1...	23	23	0	100 00	.00
36-3-3...	289	283	6	97 92	2 08	41-2-2...	29	27	2	93 10	6 90
36-3-4...	56	50	6	89.29	10 71	41-2-3...	33	28	5	84 85	15 15
37-1-1...	70	69	1	98 57	1 43	41-2-4...	22	17	5	77 27	22 73
37-1-2...	97	89	8	91.75	8 25	41-2-5...	214	206	8	96 26	3 74
37-2-1...	110	103	7	93 64	6 36	41-2-6...	23	19	4	82 61	17 39
37-2-2...	5	4	1	80 00	20 00	41-2-7...	182	179	3	98 35	1 65
37-2-3...	8	6	2	75 00	25 00	41-2-8...	22	19	3	86 36	13 64
37-2-4...	12	12	0	100 00	.00	41-2-9...	133	127	6	95.49	4 51
37-2-5...	3	2	1	66 67	33 33	41-2-10...	136	133	3	97 79	2 21
37-2-6...	7	5	2	71.43	28 57	41-2-11...	204	199	5	97.55	2 45
37-2-7...	10	9	1	90.00	10 00						
37-2-8...	4	2	2	50 00	50 00	Total.	4, 235	3, 616	619	85 38	14 62

MICHIGAN ROBUST × STRINGLESS GREEN REFUGEE

44-1-1...	199	176	23	88 44	11.56	44-3-3...	48	32	16	66 67	33.33
44-1-2...	126	124	2	98 41	1.59	44-3-4...	11	6	5	54.55	45.45
44-1-3...	124	105	19	84 68	15 32	44-3-5...	13	7	6	53.85	46.15
44-1-4...	99	80	19	80 81	19 19	44-3-6...	8	8	0	100.00	.00
44-1-5...	82	82	0	100 00	.00	44-3-7...	14	10	4	71.43	28.57
44-1-6...	9	7	2	77 78	22.22	44-3-8...	4	4	0	100.00	.00
44-1-7...	6	3	3	50 00	50 00	44-3-9...	7	4	3	57 14	42 86
44-1-8...	3	2	1	66 67	33 33	44-3-10...	4	2	2	50.00	50 00
44-1-9...	4	4	0	100.00	.00	44-3-11...	8	6	2	75.00	25 00
44-1-10...	17	14	3	82 35	17 65	44-3-12...	6	6	0	100.00	.00
44-1-11...	6	4	2	66 67	33 33	44-4-1...	30	28	2	93 33	6 67
44-1-12...	16	9	7	56.25	43 75	44-4-2...	33	28	5	84.85	15 15
44-1-13...	8	5	3	62 50	37 50	44-4-3...	132	107	25	81 06	18 94
44-2-1...	18	14	4	77 78	22 22	44-4-4...	7	3	4	42.86	57 14
44-2-2...	13	7	6	53 85	46 15	44-4-5...	5	3	2	37.50	62.50
44-2-3...	8	6	2	75.00	25 00	44-4-6...	5	5	0	100 00	.00
44-2-4...	8	5	3	62 50	37 50	44-4-7...	6	6	0	100 00	.00
44-2-5...	7	5	2	71.43	28.57	44-4-8...	5	2	3	40.00	60 00
44-2-6...	14	12	2	85 71	14 29	45-1-1...	22	14	8	63 64	36 36
44-2-7...	8	8	0	100 00	.00	45-1-2...	35	10	25	28 57	71 43
44-2-8...	11	7	4	63.64	36 36	45-1-3...	39	12	27	30.77	69 23
44-2-9...	5	4	1	80 00	20 00	45-1-4...	14	12	2	85 72	14 28
44-2-10...	6	6	0	100.00	.00	45-1-5...	17	14	3	82.35	17 65
44-2-11...	7	6	1	85 72	14 29	45-1-6...	10	5	5	50.00	50 00
44-2-12...	8	4	4	50 00	50 00	45-1-7...	8	3	5	37.50	62.50
44-2-13...	11	8	3	72 73	27 27	46-4-1...	11	5	6	45.45	54 55
44-2-14...	9	5	4	55 56	44 44	47-2-1...	7	6	1	85.71	14 29
44-2-15...	8	6	2	75 00	25 00	47-2-2...	7	4	3	57 14	42 86
44-2-16...	16	9	7	56 25	43 75	47-2-3...	4	2	2	50.00	50 00
44-2-17...	7	4	3	57 14	42.86	47-2-4...	12	12	0	100 00	.00
44-2-18...	5	5	0	100.00	.00	47-2-5...	6	6	0	100 00	.00
44-2-19...	6	6	0	100 00	.00						
44-3-1...	45	23	22	51.11	48.89	Total.	1, 488	1, 159	329	77.89	22.11
44-3-2...	28	22	6	78.57	21 43						

Table 3 shows the results of inoculating  $F_3$  progenies derived from resistant  $F_2$  plants from the cross susceptible (Stringless Green Refugee)  $\times$  resistant (Michigan Robust). The following distribution of 50  $F_3$  progenies was obtained: In 6 (12 percent) of the progenies, all plants were resistant; in 13 (26 percent), 95 to 99 percent of the plants were resistant; in 20 (40 percent), 75 to 94 percent were resistant; in 7 (14 percent), 50 to 74 percent were resistant; and in 4 (8 percent), less than 50 percent were resistant. In this case 38 percent (19 out of 50) of the progenies contained more than 95 percent resistant plants, and 40 percent (20 out of 50) contained from 75 to 94 percent resistant plants.

There were 65  $F_3$  progenies from resistant  $F_2$  plants of the reciprocal cross (Michigan Robust  $\times$  Stringless Green Refugee) available for testing. The results of inoculating these progenies with the mosaic virus are presented in table 3. The distribution in this case is markedly different from the distribution in the material described above. Thirteen (20 percent) of the progenies were 100 percent resistant, 1 (1.54 percent) was 95 to 99 percent resistant, 19 (29 percent) were 75 to 94 percent resistant, 25 (38 percent) were 50 to 74 percent resistant, and 7 (11 percent) contained less than 50 percent resistant plants.

Of the 4,235 plants tested ( $F_3$  susceptible  $\times$  resistant from plants resistant in  $F_2$ ) 3,616 were healthy and 619 were diseased, a ratio of 85.38 percent resistant to 14.62 percent susceptible. Of the 1,488 plants tested of the reciprocal cross ( $F_3$  resistant  $\times$  susceptible from plants resistant in  $F_2$ ) 1,159 were healthy and 329 diseased, a ratio of 77.89 percent resistant to 22.11 percent susceptible.

Table 4 (a summary of table 3) gives the percentages of  $F_3$  progenies, obtained from resistant  $F_2$  plants, which fall into the various divisions arbitrarily based upon the percentage of resistant plants in a progeny.

TABLE 4.—Summary of table 3

F <sub>3</sub> plants resistant (percent)	F <sub>3</sub> progenies resistant (from F <sub>2</sub> resistant plants of indicated cross)	
	Susceptible $\times$ resistant	Resistant $\times$ susceptible
	Percent	Percent
95 to 100.....	38	22
75 to 100.....	78	51
50 to 100.....	92	89
0 to 50.....	8	11

From the results obtained in the  $F_1$  and  $F_2$  generations of crosses between Michigan Robust and Stringless Green Refugee varieties it might be expected that the resistant plants from the Michigan Robust  $\times$  Stringless Green Refugee cross would produce and maintain a high degree of resistance, but this is apparently not the case. The progeny test indicates that the  $F_2$  plants of the cross Stringless Green Refugee  $\times$  Michigan Robust that were designated as resistant differed in composition from at least some of the resistant plants from the reciprocal cross.

In table 5 are presented the results of inoculating  $F_3$  progenies of plants of the Stringless Green Refugee  $\times$  Michigan Robust cross that were susceptible in the  $F_2$  generation.

TABLE 5.—Reaction to common bean mosaic of  $F_3$  generation of susceptible (Stringless Green Refugee)  $\varnothing \times$  resistant (Michigan Robust)  $\sigma$ , derived from plants susceptible in  $F_2$

STRINGLESS GREEN REFUGEE × MICHIGAN ROBUST											
Progeny no.	F <sub>3</sub> plants					Progeny no	F <sub>3</sub> plants				
	Number			Percent			Number			Percent	
	Tested	Healthy	Diseased	Healthy	Diseased		Tested	Healthy	Diseased	Healthy	Diseased
40-1-1...	288	17	271	5.90	94.10	40-4-1...	169	11	158	6.51	93.49
40-1-2...	148	9	139	6.08	93.92	40-4-2...	115	3	112	2.61	97.39
40-1-3...	98	2	96	2.04	97.96	40-4-3...	120	3	117	2.50	97.50
40-1-4...	66	1	65	1.51	98.49	40-4-4...	49	5	44	10.20	89.80
40-1-5...	70	1	69	1.43	98.57	40-4-5...	59	4	55	6.78	93.22
40-1-6...	87	0	87	.00	100.00	40-4-6...	131	10	121	7.63	92.37
40-1-7...	40	2	38	5.00	95.00	40-4-7...	32	3	29	9.37	90.63
40-1-8...	44	4	40	9.09	90.91	41-1-9...	21	1	20	4.76	95.24
40-1-9...	61	2	59	3.28	96.72	Total.	1,627	79	1,548	4.86	95.14
40-1-10...	16	0	16	.00	100.00						
40-1-11...	13	1	12	7.69	92.31						
MICHIGAN ROBUST × STRINGLESS GREEN REFUGEE											
44-1-6...	36	1	35	2.78	97.22	44-4-4...	43	17	26	39.53	60.47
44-1-7...	18	2	16	11.11	88.89	44-4-5...	33	3	30	9.09	90.91
44-1-8...	131	24	107	18.32	81.68	45-1-4...	34	4	30	11.76	88.24
44-1-9...	25	10	15	40.00	60.00	45-1-5...	24	8	16	33.33	66.67
44-1-10...	16	6	10	37.50	62.50	Total.	451	127	324	28.16	71.84
44-3-4...	91	52	39	57.14	42.86						

Of the 19 progenies tested, 2 (10 percent) were 100 percent susceptible, 8 (42 percent) were 95 to 99 percent susceptible, and 9 (47 percent) contained 90 to 94 percent of susceptible plants. These results are in close agreement with what would be expected from susceptible  $F_2$  plants. Out of 1,627 plants tested, 79 were healthy and 1,548 were diseased, i. e., 4.86 percent were resistant and 95.14 percent were susceptible.

Ten progenies derived from  $F_2$  susceptible plants of the cross Michigan Robust  $\times$  Stringless Green Refugee (table 5) gave the following distribution: 1 progeny contained 97 percent of susceptible plants, 1 contained 91 percent, 3 contained 80 to 90 percent, and 5 contained 43 to 67 percent of susceptible plants. Out of 451 plants tested 127 were healthy and 324 were diseased, i. e., 28.16 percent were resistant and 71.84 percent were susceptible.

It is evident from these results that  $F_3$  progenies derived from susceptible  $F_2$  individuals of the cross resistant  $\times$  susceptible contained more resistant plants than  $F_3$  progenies derived from susceptible  $F_2$  plants of the reciprocal cross.

In table 6 are presented the results obtained when progenies were kept disease-free in the  $F_1$  and  $F_2$  generations and inoculated with the common bean mosaic virus in the  $F_3$  generation.

TABLE 6.—Reaction to common bean mosaic of  $F_3$  generation of susceptible (*Stringless Green Refugee*) ♀ × resistant (*Michigan Robust*) ♂ and reciprocal, derived from plants uninoculated in  $F_1$  and  $F_2$ 

## STRINGLESS GREEN REFUGEE × MICHIGAN ROBUST

Progeny no.	F <sub>3</sub> plants					Progeny no	F <sub>3</sub> plants				
	Number			Percent			Number			Percent	
	Tested	Healthy	Dis-eased	Healthy	Dis-eased		Tested	Healthy	Dis-eased	Healthy	Dis-eased
35-1-1...	6	0	6	0 00	100 00	37-2-2...	7	1	6	14 28	85 72
35-1-2...	14	2	12	14. 29	85. 71	37-2-3...	10	8	2	80. 00	20 00
35-1-3...	12	0	12	. 00	100 00	39-1-1...	42	0	42	. 00	100. 00
35-1-4...	8	2	6	25. 00	75. 00	39-1-2...	8	0	8	. 00	100 00
35-1-5...	12	0	12	. 00	100. 00	39-1-3...	4	0	4	. 00	100 00
35-1-6...	4	0	4	. 00	100 00	39-1-4...	2	0	2	. 00	100 00
36-1-1...	10	2	8	20. 00	80 00	39-1-5...	25	1	24	4 00	96 00
36-1-2...	8	0	8	. 00	100 00	39-1-6...	21	1	20	4. 76	95 24
36-1-3...	2	0	2	. 00	100. 00	39-1-7...	16	0	16	. 00	100 00
36-1-4...	8	2	6	25. 00	75. 00	39-2-1...	9	0	9	. 00	100. 00
36-2-1...	12	0	12	. 00	100. 00	39-2-2...	10	1	9	10. 00	90 00
36-2-2...	22	6	16	27. 27	72. 73	39-2-3...	11	0	11	. 00	100 00
36-2-3...	12	2	10	16. 67	83. 33	39-2-4...	18	2	16	11. 11	88 89
36-2-4...	10	8	2	80 00	20 00	41-2-1...	19	1	18	5. 26	94 74
36-2-5...	12	2	10	16. 67	83. 33	41-2-2...	35	1	34	2 86	97 14
36-3-1...	16	2	14	12. 50	87. 50	41-2-3...	9	0	9	. 00	100 00
36-3-2...	18	0	18	. 00	100 00						
36-3-3...	28	2	26	7 14	92 86	Total	492	46	446	9 35	90. 65
36-3-4...	26	0	26	. 00	100. 00	F <sub>2</sub> result.				1. 27	98 73
37-2-1...	6	0	6	. 00	100. 00	F <sub>1</sub> result.				. 00	100 00

## MICHIGAN ROBUST × STRINGLESS GREEN REFUGEE

44-2-1...	8	4	4	50.00	50.00	45-4-1...	9	0	9	0.00	100.00
44-2-2...	10	6	4	60.00	40.00	45-4-2...	11	2	9	18.18	81.82
44-2-3...	4	2	2	50.00	50.00	45-4-3...	9	5	4	55.56	44.44
44-2-4...	3	1	2	33.33	66.67	45-4-4...	6	0	6	.00	100.00
44-3-1...	7	3	4	42.86	57.14	47-2-1...	8	2	6	25.00	75.00
44-3-2...	8	2	6	25.00	75.00	47-2-2...	14	3	11	21.43	78.57
44-3-3...	11	7	3	70.00	30.00	47-2-3...	22	10	12	45.45	54.55
44-4-1...	13	7	6	53.85	46.15	47-2-4...	9	3	6	33.33	66.67
44-4-2...	6	2	4	33.33	66.67	47-2-5...	8	2	6	25.00	75.00
45-1-1...	8	1	7	12.50	87.50						
45-1-2...	9	5	4	55.56	44.44	Total	213	74	139	34.74	65.26
45-1-3...	6	4	2	66.67	33.33	$F_2$ result.				55.67	44.33
45-1-4...	14	2	12	14.29	85.71	$F_1$ result.				82.35	17.65
45-1-5...	11	1	10	9.09	90.91						

Table 6 gives the data obtained from 36 tested  $F_3$  progenies of susceptible × resistant crosses. Eighteen (50 percent) of the progenies were 100 percent susceptible, 5 (14 percent) were 91 to 99 percent susceptible, 7 (19 percent) were 81 to 90 percent susceptible, 4 (11 percent) were 71 to 80 percent susceptible, and 2 (6 percent) were 20 percent susceptible. The last two progenies mentioned were the only ones that gave any considerable number of resistant plants. Of 492 plants tested, 446 (91 percent) were susceptible and 46 (9 percent) were resistant.

Twenty-three  $F_3$  progenies of the reciprocal cross (table 6) gave the following distribution: Two (9 percent) of the progenies were 100 percent susceptible, 1 (4 percent) was 91 percent, 3 (13 percent) were from 80 to 89 percent, 4 (17 percent) were from 70 to 79 percent, 3 were from 60 to 69 percent, 4 were from 50 to 59 percent, 4 were from 40 to 49 percent, and 2 were from 30 to 39 percent susceptible. Of the 213 plants tested, 74 (35 percent) were resistant and 139 (65 percent) were susceptible.

percent) were susceptible. These results tend to verify the  $F_1$  and  $F_2$  results indicating that the crosses involving susceptible maternal parents produce a preponderance of susceptible offspring, and vice versa. When no inoculations were made until the  $F_3$  generation the effect of the great-grandmaternal parent was still very evident though not so pronounced as in the  $F_1$  and  $F_2$  generations. It is of interest to note how the effect of the original maternal parent diminishes in each succeeding generation when the progenies are kept disease-free until they reach the generation which is to be studied. This is clearly shown in the results given in table 7.

TABLE 7.—Summary of tables 1, 2, and 6

Generation	Susceptible $\times$ resistant		Resistant $\times$ susceptible	
	Resistant	Susceptible	Resistant	Susceptible
	Percent	Percent	Percent	Percent
$F_1$ .....	0.00	100.00	82.35	17.65
$F_2$ .....	1.27	98.73	55.67	44.33
$F_3$ .....	9.35	90.65	34.74	65.26

It is readily apparent, from the results shown in table 7, that the reciprocal crosses behave very differently. When the susceptible variety was used as the female parent the progenies remained over 90 percent susceptible even in the  $F_3$  generation; however, the reciprocal cross was not nearly so stable in regard to mosaic resistance. The progenies changed from 82 percent of resistant plants in the  $F_1$  generation to 35 percent of resistant plants in the  $F_3$  generation. As may be seen from these results, there is a convergence of the reactions from generation to generation. The cross susceptible  $\times$  resistant converges slowly, whereas the reciprocal (resistant  $\times$  susceptible) converges more rapidly. It might be possible that the reciprocal crosses would reach the same equilibrium at some point in succeeding generations.

#### BEHAVIOR OF CORBETT REFUGEE $\times$ STRINGLESS GREEN REFUGEE AND RECIPROCAL

Corbett Refugee  $\times$  Stringless Green Refugee gave all resistant  $F_1$  plants, five individuals being tested. When the susceptible variety (Stringless Green Refugee) was used as the female parent the  $F_1$  plants were again resistant. Extracts of the 10 plants tested, inoculated to disease-free susceptible plants, gave negative results, indicating that these plants were probably not carrying the virus in a masked condition.

The results obtained in the  $F_2$  generation of Corbett Refugee  $\times$  Stringless Green Refugee show that of the 124 plants tested 113 were healthy and 11 were diseased, a ratio of 91.13 percent resistant to 8.87 percent susceptible. In the reciprocal cross (Stringless Green Refugee  $\times$  Corbett Refugee) (table 8) there were 269 healthy and 73 diseased in the 342 plants tested, or 78.65 percent resistant to 21.34 percent susceptible.

TABLE 8—Reaction to the common bean mosaic of  $F_2$  generation of resistant (Corbett Refugee) ♀ × susceptible (Stringless Green Refugee) ♂ and reciprocal

## CORBETT REFUGEE × STRINGLESS GREEN REFUGEE

Progeny no	F <sub>2</sub> plants					Progeny no	F <sub>2</sub> plants				
	Number			Percent			Number			Percent	
	Tested	Healthy	Dis- eased	Healthy	Dis- eased		Tested	Healthy	Dis- eased	Healthy	Dis- eased
209-1----	30	26	4	86 67	13 33	309-3----	2	2	0	100 00	0 00
209-2----	52	45	7	86 54	13 46	Total..					
309-1----	19	19	0	100 00	.00		124	113	11	91 13	8 87
309-2----	21	21	0	100 00	.00						

## STRINGLESS GREEN REFUGEE × CORBETT REFUGEE

210-1----	52	32	20	61 54	38 46	313-1----	31	26	5	83 87	16 13
210-2----	44	28	16	63 64	36 36	313-2----	52	45	7	86 54	13 46
210-3----	51	38	13	74 51	25 49	314-1----	14	14	0	100 00	00
311-1----	20	18	2	90 00	10 00	314-2----	23	23	0	100 00	00
311-2----	10	10	0	100 00	00	Total..					
312-1----	45	35	10	77 78	22 22		342	269	73	78 65	21 35

The reciprocals in this cross are unlike, but the dissimilarity is not as great as it is when Michigan Robust is used as the resistant parent. The results are still apparently non-Mendelian. They do not conform to any Mendelian ratio, although the results obtained when Stringless Green Refugee is used as the female parent do approach a 3 : 1 ratio (observed, 79 : 21 percent; calculated, 75 : 25 percent).

## BEHAVIOR OF CORBETT REFUGEE × MICHIGAN ROBUST AND RECIPROCAL

Crosses between the two resistant varieties have yielded all resistant  $F_1$  plants. Eleven plants from the cross Michigan Robust × Corbett Refugee and five plants from the cross Corbett Refugee × Michigan Robust have been tested. The  $F_1$  plants were self-pollinated to obtain material for an  $F_2$  generation.

Table 9 shows the results obtained in the  $F_2$  generation of Michigan Robust × Corbett Refugee. There were 729 plants tested, 663 being healthy and 66 becoming diseased (91 and 9 percent, respectively). In the reciprocal cross (table 9) 374 plants were tested, 349 being resistant and 25 being susceptible (93 and 7 percent, respectively).

The reciprocal crosses in this case reacted similarly to inoculations with the virus. However, the occurrence of susceptible plants in these crosses is unexpected, and if an explanation is possible for such facts it should help to make the findings reported in this paper more understandable.

TABLE 9.—*Reaction to common bean mosaic of F<sub>2</sub> generation of resistant (Michigan Robust) ♀ × resistant (Corbett Refugee) ♂ and reciprocal*

## MICHIGAN ROBUST × CORBETT REFUGEE

Progeny no.	F <sub>2</sub> plants					Progeny no	F <sub>2</sub> plants				
	Number			Percent			Number			Percent	
	Tested	Healthy	Dis-eased	Healthy	Dis-eased		Tested	Healthy	Dis-eased	Healthy	Dis-eased
301-1.....	166	147	19	88.55	11.45	304-2.....	40	40	0	100.00	0.00
301-2.....	86	80	6	93.02	6.98	305-1.....	37	34	3	91.89	8.11
302-1.....	154	143	11	92.86	7.14	305-2.....	42	36	6	85.71	14.29
302-2.....	65	61	4	93.85	6.15	305-3.....	26	23	3	88.46	11.54
303-1.....	49	48	1	97.96	2.04	Total..	729	663	66	90.95	9.05
303-2.....	31	23	8	74.19	25.81						
304-1.....	33	28	5	84.85	15.15						

## CORBETT REFUGEE × MICHIGAN ROBUST

306-1.....	100	95	5	95.00	5.00	308-2.....	13	13	0	100.00	0.00
306-2.....	134	126	8	94.03	5.97	Total..	374	349	25	93.32	6.68
307-1.....	105	94	11	89.52	10.48						
308-1.....	22	21	1	95.45	4.55						

## NATURE OF THE RESISTANCE

With the object of studying the nature of immunity in the resistant varieties, two plants of the Stringless Green Refugee variety just coming into blossom in the greenhouse were inoculated with juice extracted from Michigan Robust plants and two individuals were treated with juice extracted from Corbett Refugee plants. The seed of these plants was harvested and planted in the greenhouse bench. The seedlings were treated at 10-day intervals. Plants 1 to 8 were inoculated in the two-leaf stage with juice extracted from Michigan Robust plants, and plants 9 to 12 were inoculated with juice extracted from Corbett Refugee. After 10 days the second treatment was given. Plants 1 to 4 were then inoculated with the mosaic virus, and plants 5 to 8 were reinoculated with juice from the resistant Michigan Robust. Likewise, plants 9 and 10 were inoculated with virus and plants 11 and 12 were reinoculated with Corbett Refugee juice. In the third treatment, plants 5 and 6 were given the virus and plants 7 and 8 were given additional Michigan Robust juice; plant 11 was inoculated with the virus extract and plant 12 was reinoculated with juice from Corbett Refugee. The fourth treatment, given 30 days after the first, consisted of inoculating plants 7, 8, and 12 with extract from mosaic-diseased plants. All the plants developed mosaic symptoms within a short period following inoculation with the virus. These results suggest that the immunity of the Michigan Robust and Corbett Refugee varieties is not transferred to susceptible varieties by this treatment.

The effect of grafting was also studied. Reciprocal grafts were made between Michigan Robust and Stringless Green Refugee, and between Corbett Refugee and Stringless Green Refugee, whip grafts, cleft grafts, and inarching being employed. The results of this experiment are shown in table 10. In no case was the reaction of the scion altered by contact with the stock. Likewise, foliage produced on the stock



was not changed by the presence of a scion from a different variety. Both symbionts reacted to the virus as if they were growing as separate plants.

TABLE 10—*Results of grafting*

Stock	Scion	Type of graft	Reaction of scion to virus
Michigan Robust .....	Stringless Green Refugee.....	Inarch.....	Susceptible
Do.....	do.....	Whip.....	Do
Do.....	do.....	do.....	Do
Do.....	do.....	Cleft.....	Do
Stringless Green Refugee.....	Michigan Robust.....	Whip.....	Resistant
Do.....	do.....	Cleft.....	Do
Corbett Refugee.....	Stringless Green Refugee.....	Inarch.....	Susceptible
Do.....	do.....	Cleft.....	Do
Do.....	do.....	do.....	Do
Do.....	do.....	do.....	Do
Stringless Green Refugee.....	Corbett Refugee.....	do.....	Resistant
Do.....	do.....	do.....	Do
Do.....	do.....	Whip.....	Do

## DISCUSSION

It is evident that the results reported here cannot be accounted for by a simple Mendelian explanation. In Mendelian types of inheritance reciprocal crosses usually react alike. The exceptions are cases of sex linkage, etc., which have no bearing on the present problem. In examining the results obtained upon inoculating progenies from Stringless Green Refugee  $\times$  Michigan Robust crosses and reciprocals, it is found that the maternal parent determines to a large extent the reaction of the hybrid individuals. In the  $F_1$  generation this influence is very marked; in the  $F_2$  generation the effect is still very noticeable; in the  $F_3$  generation the effect is not as marked as in the preceding generations but is still conspicuous. However, the effect of the pollen parent must not be overlooked. The influence of the paternal parent was evident in 3 out of 17 plants tested in the  $F_1$  generation of Michigan Robust  $\times$  Stringless Green Refugee; it was not shown in the reciprocal cross in  $F_1$ . The effect of the pollen parent was more evident in all cases in the  $F_2$  and  $F_3$  generations.

$F_1$  plants from the cross Stringless Green Refugee  $\times$  Corbett Refugee manifest the influence of the pollen parent in a significant way.  $F_2$  individuals from reciprocal crosses between Stringless Green Refugee and Michigan Robust show a preponderant influence of the grandmaternal parent. Likewise, when Corbett Refugee was used as the resistant parent more resistant  $F_2$  individuals were obtained if the Corbett Refugee plants had been used as grandmaternal parents.

To explain the results obtained in reciprocal crosses between Michigan Robust and Stringless Green Refugee it is necessary to assume a cytoplasmic or extranuclear influence. It is also necessary to assume that a portion of the cytoplasm or particular inclusions accompanies the male gametes at the time of fertilization. The suggestion that biparental cytoplasmic inheritance occurs in the bean is substantiated by the results obtained in the writer's studies on a variegated leaf character (15).

In order to obtain independent evidence of the role played by the cytoplasm in heredity in beans, two distinct chlorophyll-deficient types were investigated. One of these, termed "yellow spot", was found

to behave in a simple Mendelian manner (14); the other, called "variegated", provided direct evidence of inheritance through the cytoplasm (15). Below is a brief summary and discussion of the results with the variegated character.

All  $F_1$  plants from the variegated  $\times$  normal green cross were variegated. One out of eight  $F_1$  plants from the reciprocal cross (normal green  $\times$  variegated) showed one small variegated area; the remaining seven plants were green.

In the second generation of the variegated  $\times$  normal green cross, 84 percent variegated and 16 percent normal green plants were obtained. In the reciprocal (normal green  $\times$  variegated) 93 percent of normal green and 7 percent of variegated plants were found. These results suggest that the variegated condition is governed by the character of the cytoplasm and that the causal agency is introduced with the pollen tube at the time of fertilization. The production of variegated offspring by plants normal green in  $F_1$  can be explained by assuming a segregation or sorting out of the material which causes the condition during growth and cell division in the  $F_1$  plants and its inclusion in such proportions in some of the  $F_2$  ovules that it comes to expression in the developing individual. The occurrence of a slight variegation in a leaf of one plant in the  $F_1$  generation of normal green  $\times$  variegated might be explained by assuming that a sufficient amount of the cytoplasm (or its inclusions) was introduced into the egg from the pollen parent to eventually influence a small area of the  $F_1$  individual.

The explanation, as outlined above, is similar to that reported by Baur (1, 2) and later by Noack (11, 12, 13), for the Albomarginata variety of *Pelargonium zonale* Willd. This form, characterized by leaves with white margins, produces sometimes pure-white and sometimes pure-green branches. Reciprocal crosses between flowers on these two kinds of branches may result in variegated offspring. Baur explains the appearance of green and white tissue by assuming the presence of two kinds of plastids or plastid primordia, which are sorted out during cell division, one type of primordium giving rise to green plastids, the other to white or colorless plastids. Baur states that these plastid primordia are contributed by both parents.

Winge (21) suggests that in those cases where variegated plants occur the cytoplasm in which the plastids lie is responsible for the defect. According to Winge the two classes of non-Mendelian inheritance, maternal and biparental, are due to the fact that in some instances paternal cytoplasm is introduced into the egg with the male gametes whereas in others it is not.

Noack (10, 11, 12) also studied *Pelargonium zonale*. He obtained essentially the same results as did Baur, except in crosses with green plants. Baur reported similar results in reciprocal crosses with pure-green plants, but in the case of the material used by Noack a larger proportion of variegated plants was obtained when *zonale* plants were used as female parents. In crosses with green, different *zonale* plants gave rise to different proportions of variegated plants and also different intensities of variegation.

According to Demerec (4) neither Baur's nor Noack's conclusions have sufficient support. He states (4, p. 151):

Results identical with those obtained in the experiments would be expected if it is assumed that the *zonale* character is determined by a dominant gene which

becomes mutable (mutating to the gene for green) in crosses with green, or when the plant is heterozygous for green. Such an assumption would be supported by similar behavior observed in several other cases.

He then discusses the work of Emerson (5) on variegated pericarp in maize in which Emerson states that the mutability of the gene for variegated pericarp is increased when the gene is in a heterozygous condition.

Demerec further states (4, p. 152):

The fact, observed by Noack, that reciprocal crosses between *zonale* and green gave different results, could be explained by the assumption that the mutability of the gene for white is increased when brought by the pollen nucleus into other than maternal cytoplasm.

Essentially, Demerec's hypothesis involves the assumption that the cytoplasm exerts an indirect effect by first influencing the gene, while the earlier explanations (1, 2, 21) assume a direct influence of the cytoplasm. Demerec grants the non-Mendelian inheritance of a chlorophyll character in maize which is only transmitted through the maternal parent. It is possible that in certain forms the cytoplasm or other extranuclear material accompanies the male gamete into the egg and in such cases biparental non-Mendelian inheritance could be explained without the necessity of assuming a high mutability of the gene when in foreign cytoplasm.

The behavior of the variegated character can be explained entirely on a non-Mendelian or cytoplasmic basis, but the cytoplasmic explanation does not cover all the facts obtained in the studies on the inheritance of resistance to the mosaic virus. Apparently the immediate reaction of the plant is determined by the character of the cytoplasm, but the nature of the cytoplasm ultimately depends upon nuclear factors. Therefore, the phenomenon may be primarily nuclear though overshadowed by the effect of the cytoplasm. We must assume that certain genotypes change the reaction of the cytoplasm more rapidly than others. Such an explanation will account for the difference in reciprocals and for the convergence which occurs in the  $F_2$  and  $F_3$  generations.

Considerable irregularity occurs in all generations of the cross Stringless Green Refugee  $\times$  Michigan Robust and reciprocal. The fact that the reciprocal crosses react differently in each generation suggests a strong cytoplasmic influence on the inheritance of resistance. For example, the  $F_3$  generation of Michigan Robust  $\times$  Stringless Green Refugee showed more susceptible plants than resistant ones in the material which had been kept disease-free until that time, and, in comparison with the reciprocal cross, a higher percentage of resistant plants from plants susceptible in  $F_2$  and a higher percentage of susceptible plants from plants resistant in  $F_2$ . Throughout the studies of this cross (Michigan Robust  $\times$  Stringless Green Refugee) neither resistant nor susceptible plants have remained stable. Some resistant and some susceptible plants have been isolated which produce only resistant and susceptible plants, respectively, but the majority of the plants, as may be seen from the  $F_3$  tables, produce some plants of the opposite reaction in the following generation. This inconsistency can also be explained by assuming that the distribution or behavior of the cytoplasmic units responsible for the resistance, or governing the action of genes for resistance, is not as regular or as systematic in cell division as is the distribution of nuclear factors.

It is apparent that the two resistant varieties used in this investigation differ in the type of resistance carried or that the resistance is governed by different heritable entities in the two. At least they differ in ability to transmit this immunity in crosses with susceptible plants.

When Michigan Robust was used as the male parent and Stringless Green Refugee as the female parent all the  $F_1$  plants were susceptible and in the  $F_2$  generation 90 percent of the plants were still susceptible. However, plants that were resistant in the  $F_2$  generation maintained a high degree of resistance in the  $F_3$  generation. When Corbett Refugee was used as the male parent in crosses with Stringless Green Refugee all the  $F_1$  plants were resistant and 79 percent were resistant in the  $F_2$  generation.

The appearance of susceptible plants in the  $F_2$  generation of crosses between the two resistant varieties is further proof that these two varieties differ in their heritable bases for resistance. Corbett Refugee was developed from a single resistant plant in a field of severely infected Stringless Green Refugee beans. If it is assumed that a dominant gene mutation occurred in this case, the following explanation is possible. Let  $R$  represent the genetic factor for resistance and  $r$  the genetic factor for susceptibility. The cytoplasm of Corbett Refugee in the absence of the genes for resistance is susceptible ( $i$ ) to the virus, and the cytoplasm of Michigan Robust is immune ( $I$ ) to the virus. The assumed genetic and cytoplasmic constitution of Corbett Refugee is  $RR(i)$ , and of Michigan Robust,  $rr(I)$ . When these two varieties are crossed the  $F_1$  is resistant, owing almost if not entirely to the genetic factor  $R$  when Corbett Refugee is used as the female parent, and to the presence of the resistant factor and also resistant cytoplasm ( $I$ ) when Michigan Robust is used as the female parent. In the  $F_2$  generation, with a monohybrid segregation, we obtain 1  $RR$  : 2  $Rr$  : 1  $rr$ . However, contrary to expectation, the homozygous recessive individuals are not all susceptible, because some of these individuals have received resistant cytoplasmic factors from the Michigan Robust parent. Therefore we have a deficiency in the homozygous recessive class no matter which way the cross is made.

The above assumption that Corbett Refugee carries genetic factors for resistance does not completely explain the results obtained in the reciprocal crosses between Corbett Refugee and Stringless Green Refugee. If the genetic constitution of Corbett Refugee is  $RR$  and that of Stringless Green Refugee is  $rr$ , then the  $F_1$  should be resistant and a ratio of 3 resistant to 1 susceptible should be obtained in  $F_2$ . The  $F_1$  results obtained are as expected. In the  $F_2$  the results obtained are not in agreement with the expected results, Corbett Refugee  $\times$  Stringless Green Refugee (observed, 113 : 11; calculated on 3 : 1 basis, 93 : 31), deviating by 20 from the expected, and Stringless Green Refugee  $\times$  Corbett Refugee (observed, 269 : 73; calculated on 3 : 1 basis, 256.5 : 85.5), deviating by 12.5 from the expected, in favor of the resistant class. The numbers involved in this cross are small, and it is possible that if larger numbers were available the results would more closely approximate a 3 : 1 ratio. It is not improbable, however, that there is some other underlying reason for the occurrence of a larger number of resistant plants than was to be expected under the explanation just given.

The complete explanation and understanding of the foregoing results may be linked with an explanation and understanding of the existence of so-called "tolerant" bean varieties, or varieties which are fairly easily infected with the virus but which do not ordinarily develop symptoms that can be readily identified. It is difficult to study progenies from crosses between tolerant varieties and either susceptible or resistant varieties, because of the difficulty in distinguishing the tolerant plants in a hybrid population.

As the complex plant viroses become better understood a technique may be developed whereby crosses between tolerant and resistant (or susceptible) varieties can be studied. Such crosses should give further evidence on the method of inheritance of resistance to the common mosaic virus in the bean.<sup>7</sup>

#### SUMMARY

Reciprocal crosses between the mosaic-resistant Michigan Robust and susceptible Stringless Green Refugee varieties of beans reacted quite differently to the mosaic virus employed in these studies. It was found that the maternal parent governs to a large extent the reaction of the hybrid offspring.

In the  $F_1$  generation, all plants were susceptible from the cross Stringless Green Refugee ♀ × Michigan Robust ♂, and 82 percent were resistant from the cross Michigan Robust × Stringless Green Refugee.

The influence of the grandmaternal parent was still very evident in the  $F_2$  generation. From the cross Stringless Green Refugee × Michigan Robust, 99 percent of the  $F_2$  individuals were susceptible to mosaic, whereas 56 percent of the individuals from the reciprocal cross were resistant.

In the  $F_3$  generation the reciprocal crosses were still very different. From the cross susceptible × resistant, 91 percent of the individuals were susceptible and 9 percent were resistant. In the reciprocal cross (resistant × susceptible), 35 percent of the  $F_3$  offspring were resistant and 65 percent were susceptible. Throughout the three generations studied there is evidenced a very marked convergence of results.

By studying  $F_3$  individuals derived from plants of known reaction in  $F_2$ , it was found that some plants classified as resistant in  $F_2$  gave rise to both susceptible and resistant plants in  $F_3$ , and also that plants susceptible in  $F_2$  gave rise to some resistant plants in  $F_3$ .

These results cannot be explained on a simple Mendelian basis, because the reciprocal hybrids react differently. To explain the fact that the maternal parent determines to a large degree the reaction of the hybrid individuals it is assumed that the cytoplasm or some extranuclear inclusion governs the immediate reaction of the plant to the virus. The convergence of the results from reciprocal crosses in the  $F_2$  and  $F_3$ , however, points to the conclusion that the ultimate control is nuclear but that there is a delayed expression of the action of the genes. The further assumption is necessary that certain genotypes change the reaction of the cytoplasm more rapidly than others.

<sup>7</sup> The writer has found no parallel in the literature of plant genetics to the behavior described here for the inheritance of resistance to common bean mosaic. Since this paper was submitted for publication, however, his attention has been drawn to an apparently similar case in mice studied by Little (8). Little found a marked difference in reciprocal crosses between a high tumor line and a low tumor line, and there is an indication, at least, of convergence of the reciprocals in  $F_3$ .

Corbett Refugee, a second resistant variety, was used in crosses with susceptible Stringless Green Refugee and with resistant Michigan Robust. The  $F_1$  generation of Corbett Refugee  $\times$  Stringless Green Refugee and the  $F_1$  generation of the reciprocal were identical, all individuals being resistant. In the  $F_2$  generation there was a difference in the reciprocal crosses. From the cross Corbett Refugee  $\times$  Stringless Green Refugee 91 percent of the  $F_2$  individuals were resistant and 9 percent were susceptible. In the reciprocal cross 79 percent were resistant and 21 percent were susceptible. These results are apparently non-Mendelian also; they do not conform to any Mendelian ratio, although the results obtained when Stringless Green Refugee is used as the female parent do approach a 3 : 1 ratio.

Crosses between the two resistant varieties yielded all resistant  $F_1$  plants. In the  $F_2$  generation, however, there were 7 percent susceptibles from Corbett Refugee  $\times$  Michigan Robust and 9 percent susceptibles from Michigan Robust  $\times$  Corbett Refugee. From these results it is quite evident that the two resistant varieties differ in their bases for resistance, or at least that they differ in ability to transmit this resistance in crosses with susceptible plants.

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# RELATION OF STOMATAL FUNCTION OF WHEAT TO INVASION AND INFECTION BY LEAF RUST (*PUCCINIA TRITICINA*)<sup>1</sup>

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## INTRODUCTION

Experience in culturing leaf rust of wheat (*Puccinia triticina* Eriks.) has shown that abundant infection of the host follows inoculations made in totally dark humidity chambers. Loftfield (8)<sup>2</sup> and Hart (6) found that stomata of wheat close and remain closed in darkness, while Hart (6) reported that stem rust (*P. graminis tritici* Eriks. and Henn.) cannot enter closed stomata. If, therefore, the stomata of the wheats used in studying leaf rust reacted as did those reported by Loftfield and Hart, it would be apparent either that leaf rust, in contrast to stem rust, can enter a completely closed stoma, or that closure commonly is insufficiently complete to exclude the entry hyphae of this rust. Studies were undertaken to afford information on this and related questions. A preliminary report on these investigations has been published (4).

## MATERIAL AND METHODS

In the first section of this work the number of entries of the fungus into the host was judged indirectly by the number of pustules appearing on leaves within 10 days after inoculation. Parallel inoculation trials were conducted in a lighted and in a dark humidity chamber, which were held in a common water bath at a relatively constant temperature of  $21^{\circ}\text{C.} \pm 1^{\circ}$ . The lighted chamber received sunlight filtered through a tray of running water, with supplementary illumination from a 500-watt incandescent lamp suspended 12 inches above the plants on cloudy days.

Inoculation methods were adapted to provide as uniform distribution of inoculum as possible simultaneously over the entire series of plants used in a single experiment. In the experiments with seedlings the first leaf of each plant was used exclusively, while younger leaves were excised before inoculation. For each variety, 10 leaves were inoculated in light and 10 in darkness. The plants to be used were grouped together on the floor, and rust spores were showered upon them by shaking heavily infected plants above them at a height of 2 to 3 feet. They were atomized with water in the evening and placed in the humidity chambers for 24 hours, where they were kept continuously moist by repeated atomizing. They were then removed to the greenhouse bench to dry off either in darkness or in very weak light. Records of infection were made after about 10 days either by counting the pustules and measuring the length of each leaf or by estimating the

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<sup>2</sup> Reference is made by number (italic) to Literature Cited, p. 932.

percentage infection by the scale adopted by the United States Department of Agriculture.

Where the pustules per leaf were counted, the standard errors for an inoculation series comprising several varieties were computed by obtaining the deviations from the mean of each variety. A common standard error for each inoculation trial was calculated for each group in the dark and in the illuminated humidity chambers.

In a part of this study, entry was studied directly by microscopic examination of the epidermis of the first seedling leaf, a portion of which was stripped from both the dorsal and the ventral leaf surfaces after the inoculated leaf had been held in the humidity chambers the length of time required for spore germination and for the formation of appressoria or substomatal vesicles. The dorsal surface was more frequently used because of the greater facility in removing the epidermis. A little practice made it possible to secure strips of epidermis the width of the entire leaf and from 1 to 2 inches long. These strips were immediately immersed in absolute alcohol containing the stain fast green. Lloyd (7) showed that by this technique the stomatal apertures could be preserved exactly as they were before removal of the epidermis. This was confirmed in the present study by careful observation and counts on marked sections of leaf before and after removal and fixation of the epidermis. By application of Lloyd's technique the relation of rust entry to stomatal aperture was studied directly. Fast green, an alcohol-soluble dye, was particularly suited to the study, since it stains the fungus more deeply than the host tissue. In such preparations the stomatal aperture, appressoria, substomatal vesicles, and infecting hyphae could be clearly seen. Gibson (5) stripped off the epidermis of leaves of various plants to study the entry of rusts into species other than their own hosts, but gave no consideration to stomatal function.

The cultures of leaf rust used in this work were pure clonal lines, cultured from either single spores or single uredia. All were repeatedly tested for purity on the standard differential varieties of Mains and Jackson (9).

The following varieties of wheat were used in the infection and entry studies: Spring varieties, Hope (C. I.<sup>3</sup> 8178), Little Club (C. I. 4066), Marquis (C. I. 3641), Kota (C. I. 5878), Norka (C. I. 4377), Progress (C. I. 6902), and Webster (C. I. 3780); winter varieties, Michigan Amber selection 29-1-1-1, Gladden selection 455-1, and Kanred (C. I. 5146).

## EXPERIMENTAL RESULTS AND OBSERVATIONS

### RELATION OF LIGHT AND DARKNESS TO STOMATAL APERTURE

As shown by Loftfield (8) and confirmed in these studies, stomatal movements of the wheat plant in response to light are regular, the stomata opening only when the plants are illuminated and closing very soon in darkness. In the present study, direct observations throughout 24-hour periods were made upon the stomatal behavior of outdoor-grown seedlings of Gladden, Kota, Marquis, Michigan Amber, and Progress wheats kept continuously supplied with abundant moisture. These seedlings were subjected to complete darkness

<sup>3</sup> C. I. denotes accession number of the Division of Cereal Crops and Diseases.

and a saturated atmosphere at various times and for various periods of the day to ascertain whether the stomata ever opened in darkness under conditions of abundant soil moisture and high atmospheric humidity, which might be thought to favor such behavior. In those cases where the stomata were open when the plants were subjected to the above conditions they closed regularly within 15 minutes, although some reacted more promptly than others. Such closed stomata failed to open again in darkness, although those of the check plants in light maintained the characteristic daily response. If the stomata were closed when the plants entered the chambers they remained closed throughout their confinement in the dark. When the plants are held in darkness for 24 hours the stomata usually fail to open normally when first again exposed to light and may require considerable illumination before they resume their normal response to light. Certain stomata appear to be dead or nonfunctional. These have invariably been found to be closed.

The stomata of mature field plants of the varieties Harvest Queen, Michigan Amber, Leap, Kharkof, and Turkey were observed at 8:30 a. m. and 1 p. m. during the period from May 12 to 29, 1933, during which period there was abundant soil moisture. It was found, contrary to Loftfield's observations (8), that within a limited range all the stomata of a leaf reacted as a group, opening in like degree or closing simultaneously, according to environmental conditions, although on certain days little opening occurred.

Many other observations involving many inoculation trials have been made, but in no case have stomata been found to be open in plants kept in darkness, while in plants exposed to light in the usual manner the stomata have been found to open regularly on exposure to sunlight or to artificial illumination of sufficient intensity. The foregoing observations would seem to indicate that the stomata of the experimental plants remain closed throughout any period in which they are kept in darkness. Furthermore, it appears from microscopic observation of prepared epidermal strips and of the living leaf that when a stoma of a normal wheat plant closes there remains not even a small crevice between the guard cells through which a hypha of a rust fungus may enter without overcoming the pressure exerted by these cells. Thus, by determining the abundance of infection resulting from inoculations made under conditions of light and darkness and checking sample leaves for stomatal condition, it is possible to ascertain the relation of open and closed stomata to infection.

#### INFECTION FOLLOWING INOCULATION OF FIRST SEEDLING LEAVES HAVING OPEN OR CLOSED STOMATA

To determine whether the open or closed condition of stomata at the time of host entry by leaf rust has any effect on the abundance of infection, the following inoculations were made: The first seedling leaves of the winter wheats Gladden, Kanred, and Michigan Amber and of the spring wheats Kota, Marquis, and Progress were inoculated in the evening with each of the forms 9, 55, and 56. The plants were atomized with water and placed in a common dark humidity chamber until morning, when they were separated into two groups, one of which was placed in the lighted and the other in the dark

humidity chamber described above. The stomata in the lighted chamber were found to open in each trial, while those in the dark chamber remained closed. This simulated the condition of evening dew followed by morning opening of stomata in the light, as contrasted with continued closure of stomata. The plants were removed to the greenhouse bench the following evening.

With odds of 19 to 1 as the criterion of significance, there were only 15 out of a total of 36 comparisons in which a significant difference in number of pustules occurred (table 1). Of these 15 significant differences, 9 resulted in a greater infection in the lighted chambers and 6 in the dark chambers. Considering the fact that no open stomata have been observed on plants in the dark chambers, these data indicate no fundamental difference in the capacity of these forms of leaf rust to enter closed or open stomata of the six varieties of wheat studied. Such significant differences as were recorded appear to have been due to failure to distribute the inoculum uniformly over the experimental plants.

TABLE 1.—Effect of open and closed stomata at time of stomatal entry on infection of first seedling leaf of 6 wheat varieties by *Puccinia triticina*, physiologic forms 9, 55, and 56

Variety	Trial no.	Pustules, <sup>1</sup> under indicated stomatal condition, of physiologic form no.—								
		9			55			56		
		Stomata open	Stomata closed	Difference <sup>2</sup> ± S.E.	Stomata open	Stomata closed	Difference <sup>2</sup> ± S.E.	Stomata open	Stomata closed	Difference <sup>2</sup> ± S.E.
		Number	Number	Number	Number	Number	Number	Number	Number	Number
Gladden.....	1	11 6	11 9	-0.3±1.64	30.6	31 2	-0.6±1.66	8.0	8.6	-0.6±0.64
	2	15 0	14 1	+ 9±1.06	7 1	8.4	-1.3±.85	10 7	13.7	-3.0±.85
Kanred.....	1	11 0	9 1	+1.9±1.64	43.2	31 2	+12.0±1.66	7.6	6 2	+1.4±.64
	2	18 0	16 2	+1.8±1.06	8.9	7.6	+1.3±.85	6.2	3.7	+2.5±.85
Kota.....	1	7 4	5 9	+1.5±1.64	24.9	29 9	-5.0±1.66	4 0	4 6	-.6±.64
	2	22 1	21.3	+ 8±1.06	7.8	6.7	+1.1±.85	6.1	4.9	+1.2±.85
Marquis.....	1	14.0	15.4	-1.4±1.64	23 4	30.0	-6.6±1.66	8.1	4.6	+3.5±.64
	2	9.0	12.8	-3.8±1.06	4 5	4.3	+ 2±.85	8.3	7.8	+ 5±.85
Michigan Amber.....	1	22 5	14 9	+7.6±1.64	25 1	28 7	-3.6±1.66	8 9	6.2	+2.7±.64
	2	19 8	9.6	+1.2±1.06	5 9	6.0	-.1±.85	7.5	8.0	-.6±.85
Progress.....	1	9 1	13 8	-4.7±1.64	30.9	16.1	+14.8±1.66	12.9	11.8	+1.1±.64
	2	10 5	9 2	+1.3±1.06	6 5	6 5	0	6.5	5 0	+1.5±.85
Average.....		13 4	12.9	+ .5±.56	18.2	17.2	+ 1.0±.53	7.9	7.1	+ .8±.31

<sup>1</sup> Mean number per linear centimeter of leaf for 10 leaves.

<sup>2</sup> Open minus closed.

Since inoculations at 21° C. showed no effect of open or closed stomata on the abundance of infection, the question arises as to whether this is true at other temperatures. Therefore a group of spring wheat varieties was inoculated with form 9 under conditions of open and closed stomata as in the above experiments at the temperatures 15°, 22°, and 29°, controlled to ±1° (table 2). The trials at each temperature involved a single experiment. The abundance of infection was estimated on the scale of percentage of possible infection. Very little infection occurred at 29° with stomata either open or closed. Abundant infection took place at 15° and 22°, but there were no consistent differences in infection at either tempera-

ture between plants inoculated in the open or in the closed stomatal condition.

TABLE 2.—*Relation of temperature to infection by physiologic form 9 resulting from inoculations of first seedling leaf with stomata open and closed*

Variety	Infection under indicated stomata, condition at—					
	15° C		22° C		29° C	
	Stomata open	Stomata closed	Stomata open	Stomata closed	Stomata open	Stomata closed
	Percent	Percent	Percent	Percent	Percent	Percent
Hope <sup>1</sup> .....	35	35	—	—	—	—
Kota.....	45	25	35	60	Trace	Trace
Little Club.....	80	80	90	85	Trace	Trace
Webster.....	65	35	80	85	Trace	Trace
Norka <sup>1</sup> .....	—	—	75	90	Trace	Trace

<sup>1</sup> Hope was inoculated only at 15° and Norka only at 22° and 29°.

#### INFECTION BY SINGLE UREDIUM CULTURES THAT HAD BEEN CONTINUOUSLY PROPAGATED SINCE COLLECTION, BY INOCULATIONS IN LIGHT

The question arises as to whether all strains of *Puccinia triticina* occurring in nature are as capable of infecting when entry must occur through closed stomata as were the cultures of forms 9, 55, and 56 considered above. Since these three cultures had been propagated for several years without light during the period of stomatal entry, it seemed possible that they might have been unknowingly selected because of their ability to effect abundant entry in darkness. Therefore 15 single-uredium cultures, from collections made in 9 different States in 1932, which had been continuously propagated by inoculations in light from the time of collection, were tested for capacity to infect the first seedling leaf of Gladden, Michigan Amber, and Progress wheats having open or closed stomata during the period of stomatal entry. The conditions of open and closed stomata were brought about as in the above experiments. The results given in table 3 are the estimated percentages of infection. These cultures were found to comprise seven different physiologic forms and are fairly representative of those prevalent in the eastern part of the United States in 1932. There is no evidence that the initial open or closed condition of the stomata at the time of appressorium formation had any effect on the amount of infection by any of these cultures.

TABLE 3—Effect of open and closed stomata at time of stomatal entry on infection of first seedling leaf by 15 single-uredium cultures of *Puccinia triticina* that, after collection, had been continuously propagated by inoculation in light

Place of collection	Physiologic form no	Infection under indicated stomatal condition on—					
		Gladden		Michigan Amber		Progress	
		Stomata open	Stomata closed	Stomata open	Stomata closed	Stomata open	Stomata closed
		Percent	Percent	Percent	Percent	Percent	Percent
East Lansing, Mich. ....	9	30	30	30	30	30	30
Marion County, Ind. ....	15	25	25	50	35	75	25
Elk Creek, Va. ....	15	15	15	15	15	15	25
Statesville, N. C. ....	15	60	60	50	50	75	75
Moultrie County, Ill. ....	39	65	75	65	75	65	65
Gainesville, Fla. ....	55	75	75	60	75	75	75
State College, Pa. ....	56	30	30	50	50	40	40
Columbus, Ohio. ....	56	85	85	75	75	85	85
La Fayette, Ind. ....	56	75	75	75	75	90	50
Elkhart, Ind. ....	56	20	60	60	60	30	60
Vanderburgh County, Ind. ....	56	20	40	60	40	65	50
Raleigh, N. C. ....	56	50	50	50	50	50	50
Experiment, Ga. ....	60	50	50	50	50	50	50
Allen County, Ohio. ....	61	10	15	35	35	50	40
Fairfield County, Ohio. ....	61	15	15	50	15	45	35
Average. ....		41.7	46.7	51.7	48.7	56.0	50.3

#### INFECTION RESULTS WITH MATURE PLANTS

In extensive testing of leaf rust resistance of mature wheat plants during the winter months in the greenhouse at La Fayette, Ind., heavy infection has usually been secured. The conditions during the period of possible fungus entry were such that it seemed reasonable to assume that entry took place through closed stomata. Thus, it has been the practice to shower the plants with inoculum at about 4:30 p. m., wet them with a fine spray of water, and cover with several layers of wet muslin cloth. The cloths were removed between 8 and 9 a. m. the next day and the plants allowed to dry at once. Since more than an hour of daylight is necessary to cause stomatal opening during the winter, it appears that the stomata usually remained closed until the moisture had evaporated from the plants. Further evidence that entry was effected before the plants were uncovered was obtained with a large series of mature plants in which abundant infection occurred, even though the wet plants were heavily dusted with sulphur for mildew control immediately following removal of the muslin cloths. Other experiments relating to the control of cereal rusts by the application of sulphur dust indicate that but little infection occurs after such treatment.

Mature Kota and Marquis wheat plants, in duplicate series of 15 plants each, were inoculated with physiologic form 56 and exposed to light and to darkness at 21° C. during the period of stomatal entry. Moderately abundant infection occurred from inoculations in the light, with slightly less from those in darkness—a result not unlike that obtained with seedlings and reported above.

FACILITY OF ENTRY THROUGH INITIALLY OPEN OR CLOSED STOMATA AS DETERMINED DIRECTLY BY SUBSTOMATAL VESICLE COUNTS

In the experiments described above, the ability of *Puccinia triticina* to enter the host stomata was inferred from the abundance of infection. This method is subject to inaccuracies incident to unequal distribution of both inoculum and moisture on the leaves. These defects were eliminated almost entirely by the technique described above, wherein the epidermis was stripped from the inoculated leaves and examined directly with the microscope to determine the percentage of appressoria that had effected entry.

Triplicate trials with physiologic form 55 on Michigan Amber were made by this method. The first leaves of seedling plants grown in the greenhouse were inoculated at 2:30 a. m. to insure abundant germination following the opening of the stomata. Some of the plants were placed in the dark and some in the lighted inoculation chamber. In the latter the stomata opened wide the next day and remained so until 4 p. m., when the epidermis was stripped. In the dark chamber the stomata remained closed all day. Unpublished data by the junior writer indicate that with physiologic form 55 stomatal entry occurs most abundantly between 6 and 12 hours after inoculation. It may therefore be assumed that in this experiment the majority of appressoria developed after the stomata had opened in the lighted chamber and over closed stomata in the dark chamber. By systematic inspection of the strips of epidermis the percentages of those appressoria from which entry had occurred were determined, and the results are presented in table 4. It will be seen that on leaves with open stomata an average of 89.8 percent of the appressoria had effected entry at the time of stripping, while, on leaves with closed stomata, 85.3 percent had succeeded. The difference seems insignificant in view of the high percentage of entries.

TABLE 4.—Effect of open and closed stomata on stomatal entry by *Puccinia triticina*, physiologic form 55, in Michigan Amber wheat as determined by substomatal-vesicle counts

Inoculation trial no.	Stomata open			Stomata closed		
	Appressoria not effecting entry		Appressoria effecting entry	Appressoria not effecting entry		Appressoria effecting entry
	Number	Number		Number	Number	Percent
1.....	15	115	88.5	32	155	82.9
2.....	19	135	87.7	24	157	86.7
3.....	15	181	92.3	25	176	86.3
Total.....	49	431	89.8	84	488	85.3

MODE OF ENTRY AND ITS RELATION TO STOMATAL APERTURE

A study was made of the mode of stomatal entry into the first seedling leaf of plants of Gladden, Kanred, Kota, Marquis, Michigan Amber, and Progress wheats by 18 cultures of *Puccinia triticina*.

These cultures, collected from several different States, comprised the physiologic forms 9, 15, 39, 55, 56, 60, and 61. Epidermal strips, prepared as already described, were examined. Observations were made with respect to the entire sequence of development, from spore germination through the formation of the appressorium, the infecting hypha, and the first haustorium mother cell.

The formation of appressoria has been observed to occur exclusively over stomata. As shown by Allen (2), more than one appressorium may occupy a single stoma (fig. 1). Instead of producing a normal entry hypha, an occasional appressorium may develop a small vesicle on the surface of the leaf, similar in appearance to a substomatal vesicle. This, in turn, may produce a hypha similar in appearance to an infecting hypha, but on the leaf surface. The appressorium usually is formed upon one end of the stoma (fig. 2, A, B), but occa-

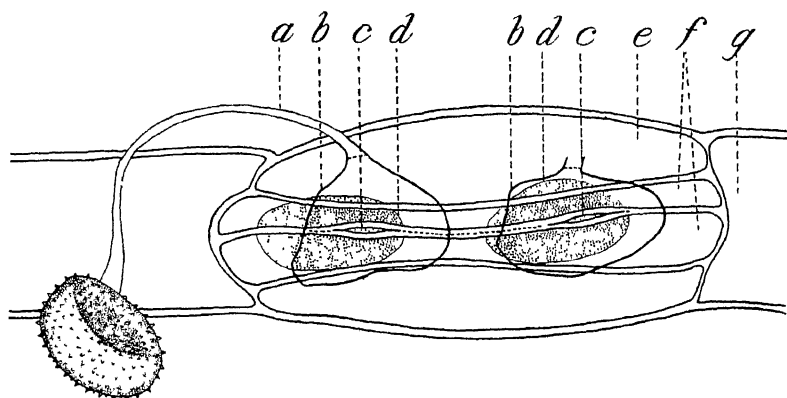


FIGURE 1.—Stoma of Michigan Amber wheat showing entry from two appressoria of *Puccinia triticina* physiologic form 55: a, Germ tube of urediospore; b, walls of empty appressoria; c, slits between guard cells forced open by entry of the rust; d, substomatal vesicles filled with protoplasm; e, accessory cell of stoma; f, guard cell of stoma, g, cell of epidermis. Drawing (semidiagrammatic) made with the aid of the camera lucida.  $\times$  #375

sionally it may be found near its center (fig. 3, B). Entrance never has been found to occur directly from a germ tube without the formation of an appressorium.

Although the appressorium may begin to form over an open stoma the latter soon closes, even before the appressorium is fully developed (fig. 2, A). Where appressoria form over closed stomata in darkness, the occupied stomata fail to open when the plants are again illuminated (fig. 2, B). In observations of hundreds of appressoria on strips of epidermis with normally open stomata, no instance has been found where a mature appressorium occupied a stoma that was more than very slightly open, and these latter cases were very rare.

Closure of stomata occupied by rust was shown to occur prior to entry in strips of epidermis of six wheat varieties inoculated with *Puccinia triticina* physiologic forms 55 and 56 (table 5). The strips were taken 6 to 8 hours after inoculation, before many entries had occurred and after exposure of the inoculated plants to light in the humidity chamber for from 3 to 4 hours. This was sufficient light to cause opening of a majority of the unoccupied stomata. On the epidermal strips the appressoria that have effected entry can be



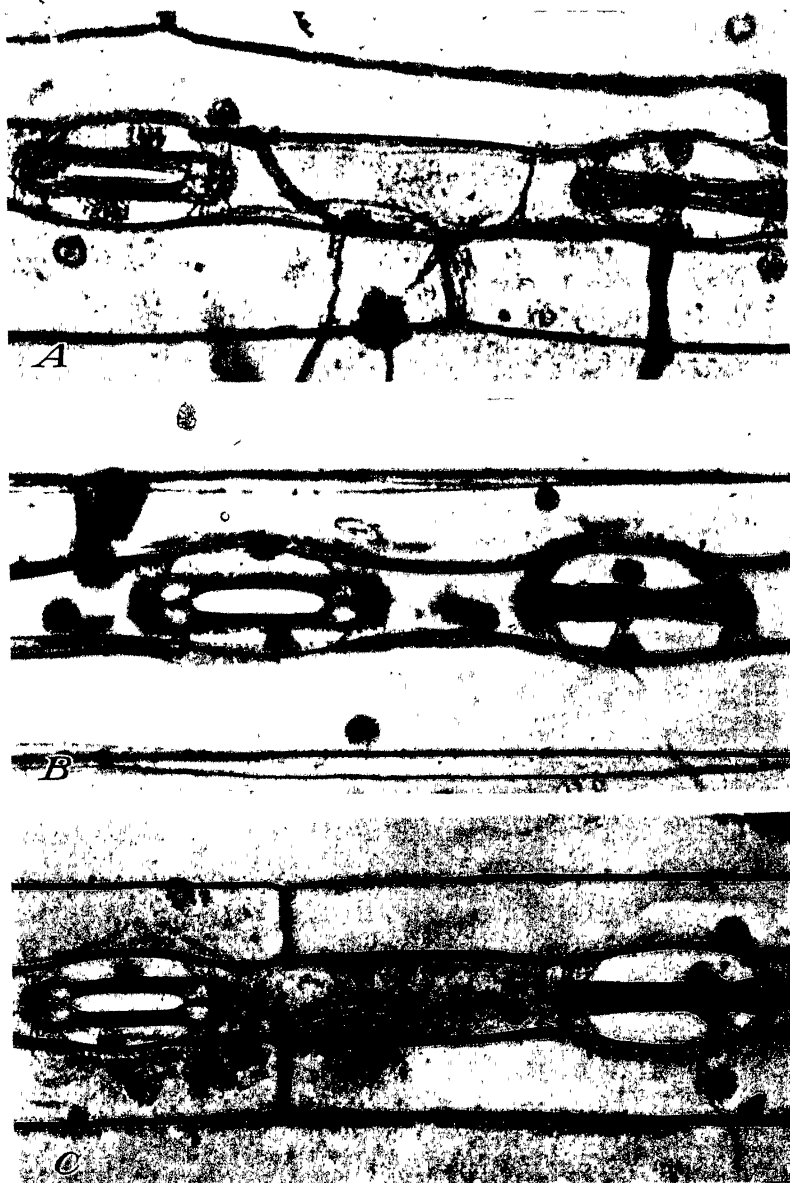


FIGURE 2.—A, At right, photomicrograph of an immature appressorium of *Puccinia triticina* physiologic form 9 over a closed stoma of the first seedling leaf of a Michigan Amber wheat plant. This appressorium probably began to form while the stoma was yet open. At left, an open, unoccupied stoma.  $\times$  about 360. B, At right, similar to A, except that the appressorium is mature and probably was formed at night over a closed stoma which failed to open in the morning. Entry has not yet occurred.  $\times$  about 395. At left, an open, unoccupied stoma. C, At right, similar to A, but with *Uromyces trifolii*  $\times$  about 395. At left, an open, unoccupied stoma.



FIGURE 3.—For explanatory legend see opposite page.

distinguished readily from those that have not by the empty appressorium wall and by the dark-staining substomatal vesicle (fig. 4, A). The stomata of all six varieties apparently reacted alike to the appressoria in closing prior to entry by the fungus. Of a total of 618 observations of individual appressoria of physiologic form 55, 615 had caused the underlying stomata to close, while 3 had permitted a very narrow slit to persist. Of the unoccupied stomata observed on the same epidermal strips, 94 percent were open. The behavior of the six wheat varieties was almost identical. Similar results were obtained from physiologic form 56.

TABLE 5.—Effect of appressoria of *Puccinia triticina* physiologic forms 55 and 56, prior to entry, on stomatal function of first seedling leaf of wheat<sup>1</sup>

Variety	Inoculated with <i>P. triticina</i> physiologic form 55						Inoculated with <i>P. triticina</i> physiologic form 56					
	Occupied stomata			Unoccupied stomata			Occupied stomata			Unoccupied stomata		
	Closed		Percent	Closed		Percent	Closed		Percent	Closed		Percent
	Number	Number		Number	Number		Number	Number		Number	Number	
Michigan Amber.....	118	0	0	28	472	94.4	164	0	0	18	882	97.4
Gladden.....	62	2	3.13	65	838	92.8	66	1	1.49	44	466	91.4
Kanred.....	75	0	0	42	658	94.0	83	0	0	93	707	88.4
Kota.....	126	1	.79	42	858	95.3	45	0	0	11	389	97.3
Marquis.....	86	0	0	49	651	93.0	28	0	0	13	387	96.8
Progress.....	148	0	0	58	942	94.2	32	0	0	22	400	94.8
Total.....	615	3	49	284	4,419	94.0	418	1	24	201	3,031	93.8

<sup>1</sup> Plants were held in illuminated humidity chambers following inoculation until the time of stripping.

Following stomatal entry and subsequent transfer of the protoplasm from the appressorium to the substomatal vesicles or infecting hyphae, the stomata still fail to open when exposed to light. In table 6 data are given from Michigan Amber plants, inoculated with physiologic form 55, from which the epidermis was stripped about 18 hours after inoculation and after unoccupied stomata had been induced to open in the light. No attempt was made to determine whether entry had been effected from each appressorium observed, but it was obvious that all but a very few appressoria were empty. Of 536 stomata occupied by appressoria and presumably entered, 535 were tightly closed while 1 possessed a narrow slit. Of 1,641 unoccupied stomata on the same strips of epidermis, 86.6 percent were open.

#### EXPLANATORY LEGEND FOR FIGURE 3

A, Photomicrograph of a stoma of the seedling leaf of Michigan Amber wheat entered by *Puccinia triticina* physiologic form 9 near one end of the stoma. a, Stomatal slit opened by the fungus; b, wall of empty appressorium; c and d, nuclei of an accessory cell and epidermal cell, respectively, that have become oriented near the point of entry. X about 707. B, Similar to A, with physiologic form 56, but entry occurred at the approximate center of the stoma. a, Wall of empty appressorium; b, stomatal slit opened by the fungus; c and d, nuclei of an accessory and epidermal cell, respectively, that have become oriented near the point of entry. X about 707. C, A normal stoma of Michigan Amber closed in darkness, no opening present. X about 707. D, A minute slit in an abnormal stoma, adjacent to that in C, in which the accessory cell has been replaced by a hair cell, preventing the normal closing of the stoma. X about 707.

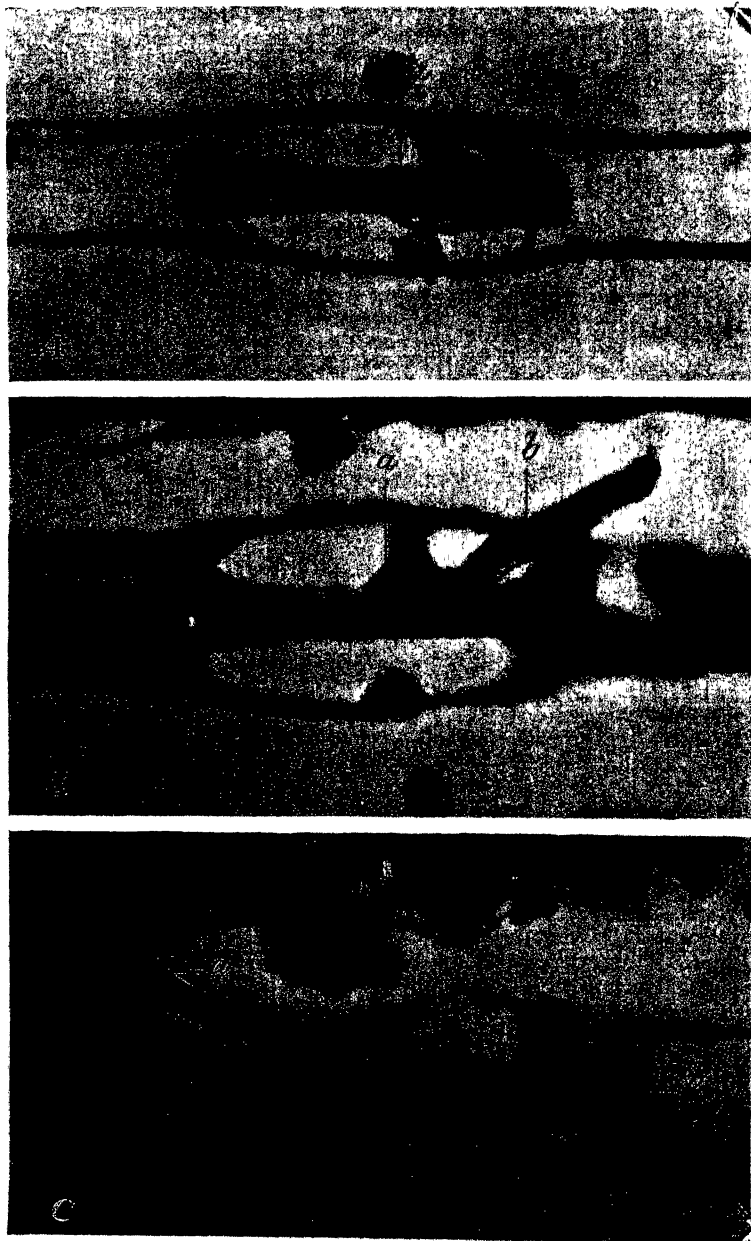


FIGURE 4.—For explanatory legend see opposite page.

TABLE 6.—Effect of *Puccinia triticina* physiologic form 55, subsequent to entry, on stomatal function of first seedling leaf of Michigan Amber wheat

Inoculation trial no.	Stomata entered			Stomata unentered		
	Stomata closed	Stomata open		Stomata closed	Stomata open	
	Number	Number	Percent	Number	Number	Percent
1.....	252	1	0.4	180	626	77.7
2.....	154	0	0	18	330	94.8
3.....	129	0	0	22	465	95.5
Total.....	535	1	.2	220	1,421	86.6

The direct observation of the effect of the living appressoria on stomata of living plants was facilitated by the use of a Leitz Ultropaque microscope. For this work wheat seedlings were grown in small glass vials and the whole was mounted on the mechanical stage of the microscope. The seedling leaves were inoculated with leaf rust and were examined after the urediospores had germinated, and the unoccupied stomata were induced to open in light. Many stomata occupied by appressoria were observed and all were completely closed, while neighboring unoccupied stomata were very largely open.

The entry hypha from the appressorium apparently exerts a pressure in pushing through between the closed guard cells. In many cases where the stomata were favorably oriented and the contents of the appressoria had moved into the substomatal vesicles or infecting hyphae, it was possible to see clearly, through the transparent appressorium, the minute lens-shaped opening between the guard cells, apparently produced by the force of the entry hypha (figs. 1 and 3, *A*, *B*). The entries shown in figures 1 and 3, *A*, are at the usual point of entry, i. e., at the ends of the stoma. Figure 3, *B*, however, shows a case where entry was effected at the center of the stoma.

The study of strips of epidermis fixed in alcohol and of the living leaf by means of the Leitz Ultropaque microscope failed to disclose in normally closed stomata any openings such as are shown below the appressoria in figures 1 and 3, *A*, *B*. Were such minute slits normally present they would be readily observed in alcohol mounts of epidermis, as is shown in figure 3, *D*. Here the accessory cell has failed to develop and has been replaced by a hair cell which inter-

## EXPLANATORY LEGEND FOR FIGURE 4

*A*, Photomicrograph from the outside of a stoma of a seedling leaf of Michigan Amber wheat. The stoma has been invaded by an entry hypha of *Puccinia triticina* physiologic form 9. Taken with a low-power objective to give depth of focus, showing the empty appressorium wall at *a*, and the deep-staining substomatal vesicle at *b*.  $\times$  about 707. *B*, As in *A*, with physiologic form 39, but viewed from the inner side of the epidermis strip. *a*, Protoplasm-filled substomatal vesicle, *b*, young infecting hypha.  $\times$  about 790. *C*, Photomicrograph of two substomatal vesicles and one infecting hypha of *P. triticina* physiologic form 15 viewed from the inner side of the epidermis of the first leaf of a Gladden wheat plant. *a*, A haustorium mother cell wedged in place against the mesophyll cells. Note that the infecting hypha grew out parallel to the epidermis. A mesophyll cell showing as a shadow over the haustorium mother cell, because above the focal plane, makes relatively certain the conclusion that the haustorium mother cell was undisturbed in the making of the preparation. *b*, Empty substomatal vesicle from which infecting hypha was produced. A portion of the infecting hypha is out of focus and shows as a shadow over the vesicle at the left.  $\times$  about 707.

fered with the normal functioning of the stoma. Other stomata on this specimen of epidermis were tightly closed, as shown in figure 3, *C*, from a photomicrograph of the stoma nearest to that shown in figure 3, *D*.

There commonly is an orientation of the nuclei of the stomatal accessory cells and adjacent epidermal cells to the wall nearest to a point of entry, suggesting a response to an injury or a chemical stimulus (figs. 3, *A*, *B*, and 4, *A*). Normally, for noninvaded stomata, the nuclei of the accessory cells lie against the wall outermost from the guard cells (fig. 3, *C*, *D*).

One infecting hypha was regularly produced, as found by Allen (3). An entire substomatal vesicle and an infecting hypha are shown in figure 4, *B*. In figure 4, *C*, is shown a haustorium mother cell wedged in the angle between mesophyll cells that have adhered to the epidermis. In this and other instances the infecting hyphae quite definitely grew out approximately parallel to the epidermis rather than at right angles, which, as Allen (1) has indicated, was characteristic for the culture of *Puccinia triticina* form 11 with which she worked. These cases may have been exceptions, since infecting hyphae extending directly into the substomatal cavity would very probably be disturbed in the preparation of the epidermal strips.

A study was made of the entry of *Uromyces trifolii* (Hedw. f.) Lév. from red clover (*Trifolium pratense* L.) into the first seedling leaf of Michigan Amber wheat. In two such inoculation experiments, reported in table 7, the behavior of this rust on wheat was identical with that of *Puccinia triticina* in readily entering the stomata, which regularly closed prior to entry (fig. 1, *c*).

TABLE 7.—Effect of appressoria of clover rust (*Uromyces trifolii*), prior to entry, on stomatal function of first seedling leaf of Michigan Amber wheat

Inoculation trial no.	Occupied stomata			Unoccupied stomata		
	Closed	Open		Closed	Open	
	Number	Number	Percent	Number	Number	Percent
1.....	241	0	0	29	180	86.1
2.....	193	0	0	17	243	93.5

## DISCUSSION

While it is not possible to generalize fully on the capacity of the leaf rust of wheat to enter closed stomata, this study indicates that it is unlikely that any of the strains of this rust occurring in the United States depend upon open stomata for host invasions. Hart (6) has described a type of resistance to stem rust (*Puccinia graminis tritici*), which she terms "functional resistance." This consists of a habit of delayed morning opening of stomata of certain wheats, preventing the entry of this rust until the moisture on the plant has evaporated, thus exposing the delicate germ tubes to desiccation and death before entry can be effected. The results of the present study would seem to dismiss the possibility of this type of resistance to leaf rust, since each of the 18 cultures of leaf rust studied was able to enter closed stomata and since forms 9, 55, and 56, studied in greater detail,

apparently never enter open stomata because of the prompt stomatal closure in response to appressorium formation.

It has been generally assumed (2, 6, 8) that entry of the cereal host by rusts occurs only through open stomata. The appressorium of the germinating urediospore has apparently been considered to have no function beyond that of a germ tube. So far as concerns the cultures of leaf rust here employed, it is highly probable that the appressorium is an organ particularly adapted to force stomatal entry, having a function somewhat similar to that of the appressorium originating from the basidiospore, which effects direct penetration of the epidermis.

Allen (1, 2) states that the appressorium probably yields some toxic secretion that exerts a harmful effect on the host cells, in some instances even killing the guard cells. Whatever the nature of this injury, it does not appear to be the sole means by which entry through closed stomata is made possible, although it may play a part in that process. The very small stomatal slit forced open by the entry hypha indicates that a definitely localized pressure is exerted on the stoma, just as happens in cases of direct penetration of the epidermis of the alternate hosts by the gametophytic phase of rusts and by other fungi effecting direct penetration.

The response of the nuclei of the stomatal accessory and epidermal cells of the host in orienting themselves against the cell walls nearest to an entering leaf rust fungus supplements Allen's observation (2, 3) on the effects of the leaf rust fungus on the position of nuclei of accessory cells and the condition of the entire protoplast of the guard cells. She noted in Malakof wheat (resistant) at least two instances in which the accessory cell nuclei lay as close as possible to the entering fungus, while in Little Club (susceptible) the staining reaction indicated that the protoplasts of the guard cells of the invaded stomata often were killed and their walls altered chemically. She (1) further observed in the case of stem rust, *Puccinia graminis tritici*, that the guard cells and adjoining epidermal cells of both susceptible and resistant varieties may be severely injured or killed by the effect of the appressorium. The orientation of the nuclei observed in the writers' studies suggests a traumatotactic response similar to that of corn seedlings injured by disease or otherwise, as reported by Pearson (10) and in other investigations reviewed by her. In the case of leaf rust, where the invasion of the cereal host occurs through a forced entry of stomata, it is possible that this injury may be a means of facilitating such entry. On the other hand, the injury may be quite unrelated to entry.

#### SUMMARY AND CONCLUSIONS

A simple technique is described for studying the entrance phenomena of a rust in relation to stomatal aperture. This involves the stripping of the inoculated epidermis from the mesophyll cells and its fixation in absolute alcohol containing a suitable stain.

The initial open condition of stomata of seedling wheat, prior to appressorium formation, is unnecessary to entry by leaf rust (*Puccinia triticina*), as represented by 18 cultures comprising 7 physiologic forms. Limited studies suggest the same relation for mature plants.

The normally closed stomata of wheat seedlings provide no openings through which entry hyphae of leaf rust can pass without overcoming the resistance exerted by the tightly closed guard cells. When an appressorium of leaf rust is initiated on an open stoma of a seedling leaf, the stoma closes tightly prior to entry.

The appressorium originating from the urediospore appears to function as a specialized organ to apply pressure between the closed guard cells, thereby effecting a forced opening of the stoma and entry by the fungus.

Since closed stomata offer no effective barrier to the entry of *Puccinia triticina*, there is no possibility of "functional resistance" to this rust through delayed morning opening of stomata.

Appressoria of clover rust (*Uromyces trifolii*) also cause wheat stomata to close and have been observed to effect numerous entries through initially closed stomata of seedling wheat leaves.

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# INHIBITING EFFECT OF SULPHUR IN SELENIZED SOIL ON TOXICITY OF WHEAT TO RATS<sup>1</sup>

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## INTRODUCTION

The recent discovery (9, 13)<sup>3</sup> that a serious disease of livestock is caused by ingestion of selenium contained in vegetation grown on certain soils of the Middle Western States led to an investigation of the absorption of selenium by plants and of the factors affecting it (10, 11). In the course of these investigations it was found that the addition of but 1 part per million of selenium as sodium selenate to the soil (Keyport clay loam) rendered wheat grown thereon toxic to white rats (14). Later it was found<sup>4</sup> that wheat grown with this concentration of selenium produced symptoms of injury similar to but not so extreme as those produced by feeding the naturally toxic wheat from the Middle West. There soon followed the discovery that the quantity of selenium absorbed by the plants could be reduced by increasing the sulphur in the soil or nutrient solution (10, 11). The bearing of this interrelationship of the two elements on the problem of control or amelioration of the disease in seleniferous soil areas was at once apparent. Experiments were therefore designed to determine whether the addition of excess sulphur to a selenized soil would reduce or prevent the toxicity of wheat grown upon it. The results of these experiments are reported in the present paper.

## METHODS

A winter wheat (Dixie, a strain of Purplestraw) was grown at the Arlington Experiment Farm, Rosslyn, Va., on eight outdoor plots of Keyport clay loam about 5 feet square separated by 8-inch buffer zones of soil between boards extending 6 inches into the ground. To six of these plots selenium was added at the rate of 2 p. p. m. as sodium selenate ( $\text{Na}_2\text{SeO}_4 \cdot 10 \text{ H}_2\text{O}$ ), calculated on the basis of a depth of 6 inches of soil (5 g sodium selenate in 1,200 pounds of soil). Two of the plots, nos. 2 and 6, received only the selenium. Plots 3 and 7

<sup>1</sup> Received for publication Feb. 1, 1936; issued July 1936.

<sup>2</sup> The writers are indebted to Hazel E. Munsell, in charge of the Foods and Nutrition Division of the Bureau of Home Economics, for general supervision of the feeding tests.

<sup>3</sup> Reference is made by number (italic) to Literature Cited, p. 841.

<sup>4</sup> The toxicity of this grain, grown in quartz sand cultures, has been studied in comparison with that of the naturally toxic grain from South Dakota by Hazel E. Munsell, Grace M. De Vaney, and Mary H. Kennedy, of the Bureau of Home Economics, and their report is being prepared for publication.

received in addition flowers of sulphur at the rate of 1,500 pounds per acre (1 pound per plot), and plots 4 and 8 received the same amount of sulphur in the form of gypsum (5 pounds per plot). Two control plots, nos. 1 and 5, received no treatment.

The sulphur and gypsum ( $\text{CaSO}_4$ ) were added first and thoroughly mixed with the soil to a depth of 6 inches. The requisite amount of sodium selenate (5.08 g, supplying 1.09 g of selenium) was added 2 days later. It was dissolved in several gallons of water in a watering can and carefully sprinkled on the soil, which was then thoroughly turned and mixed to a depth of 6 inches. The seed was sown on October 18, 1933. Some of the young plants were cut the following spring (April 18, 1934) for selenium analyses, and the rest were allowed to mature. Neither the plants nor the grain from the selenized plots differed in appearance from those of the control plots receiving no selenium, 2 p. p. m. selenium in this soil being insufficient to produce symptoms of injury (10, 11).

White rats were used to test the toxicity of the grain, since they clearly indicate the presence of small amounts of selenium in their food by an initial reluctance to eat and by pronounced tissue changes in the liver (5, 8). The grain was incorporated in the diet of the young rats when they were weaned at the age of 28 days. The grain constituted 70 percent of the diet, the rest being skim-milk powder (13 percent), dried bone and meat scrap (5 percent), yeast powder (5 percent), butter (5 percent), and cod-liver oil (2 percent). All the rats of a given experiment were of the same sex and from the same litter and were kept in individual cages and weighed weekly.

The feeding tests comprised three experiments. In the first, four female rats were fed all they would eat of the diets containing the grain from plots 1 to 4, respectively. The second experiment was a duplication of the first, with grain from plots 5 to 8 fed similarly to female rats of another litter.

Since the more selenium there is in the diet the less the rats will eat (5, 8), a third experiment was designed to distinguish between the direct effects of the selenium and the effects due to inanition from the reduced food intake. Litter-mate males were fed grain from plots 1, 3, and 4 limited in amount to that eaten during the preceding 24 hours by a rat fed the "selenium-alone" grain from plot 2. In order to determine the extent to which inanition from insufficient food supply determined the growth rates of these rats, a fifth male of the litter was fed all he would eat of the control grain from plot 1.

At the close of the experiments the rats were autopsied, and the livers especially were examined for evidence of selenium poisoning.

## RESULTS

The average weekly food consumption of the rats, their initial and final weights, and the presence or absence of liver symptoms at autopsy are given in table 1. The growth curves are presented in figures 1 and 2.

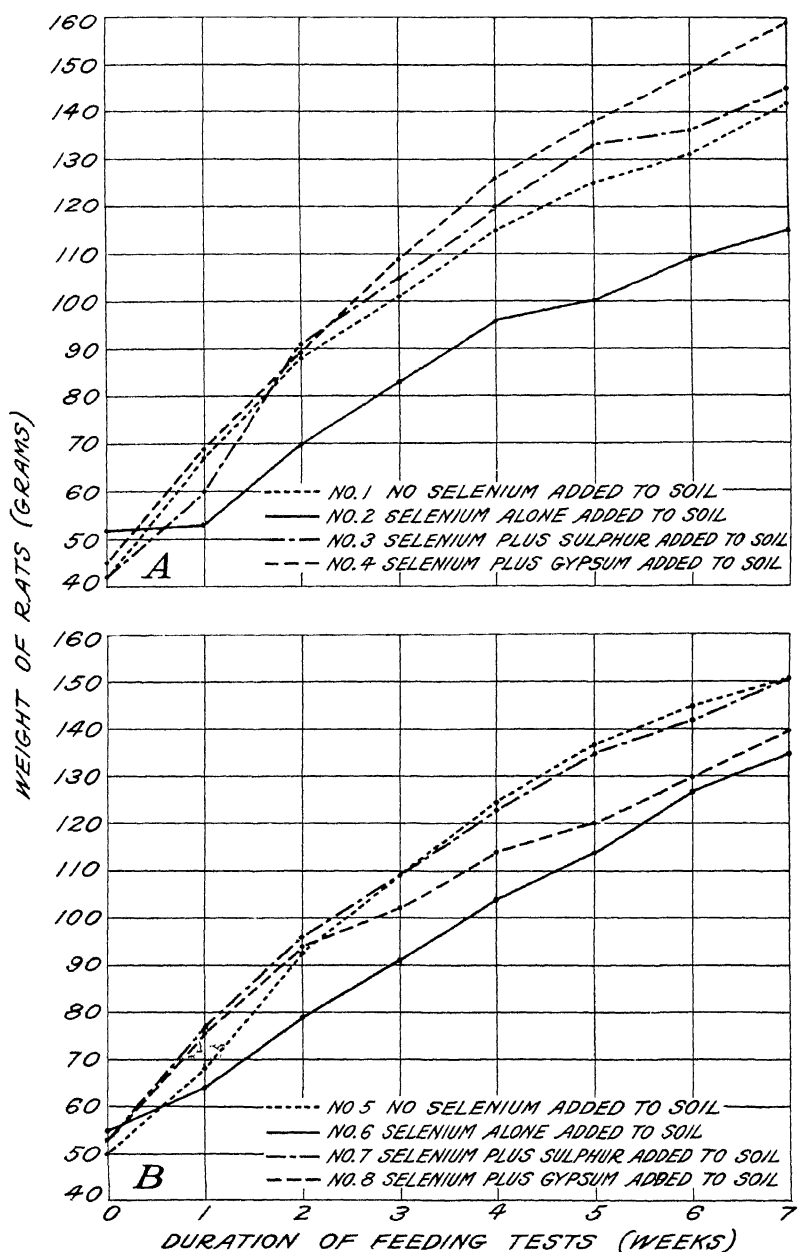


FIGURE 1.—Growth curves of female rats fed all they would eat of diets containing grain from untreated soil and from soil treated with 2 p. p. m. of selenium (as sodium selenate), with and without sulphur applications: *A*, First experiment, with grain from plots 1 to 4; *B*, second experiment, with grain from the duplicate plots 5 to 8. The feeding of the second group was continued longer than that of the first, and the final weights, not included in this graph, are given in table 1.

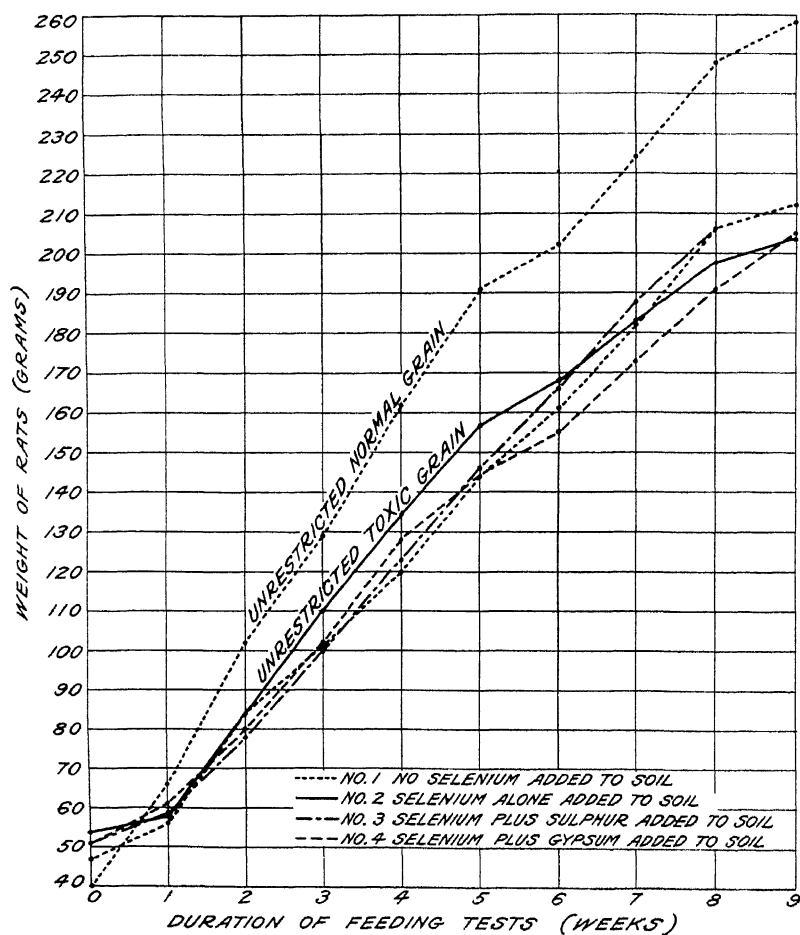


FIGURE 2—Growth curves of male rats of the third experiment, fed diets containing grain from plots 1 to 4, with the daily food intake of rats 1, 3, and 4 limited to that of the rat fed on the toxic grain from plot 2 (curve marked "unrestricted toxic grain"). The curve marked "unrestricted normal grain" is that of a male rat (no. 5) from the same litter fed all he would eat of the diet containing control grain from plot 1.

TABLE 1.—*Effect on white rats of feeding wheat grown on selenized soil with and without applications of sulphur*

Experiment and rat no.	Soil plot no.	Soil treatment		Average weekly food intake of rats (in grams) during—					Growth of rats (in grams)			Presence (+) or absence (—) of symptoms of selenium poisoning in the liver
		Selenium added in parts per million	Form of sulphur added	First week	Second to fourth weeks	Fifth to seventh weeks	Eighth to ninth weeks	Tenth to eleventh weeks	Initial weight	Final weight	Gain	
1 Unrestricted feeding												
Rat no 1.....	1	0	None.....	49.4	62.2	69.4	-----	-----	42	142	100	—
Rat no 2.....	2	2	do.....	22.6	44.1	46.6	-----	-----	52	115	63	+
Rat no 3.....	3	2	Sulphur....	40.0	68.0	63.7	-----	-----	42	145	103	—
Rat no 4.....	4	2	Gypsum....	45.1	62.9	66.4	-----	-----	45	159	114	—
2 Unrestricted feeding:												
Rat no 1.....	5	0	None.....	60.0	70.4	67.6	69.2	68.5	50	177	127	—
Rat no 2.....	6	2	do.....	42.8	49.6	58.2	61.2	62.4	55	162	107	+
Rat no 3.....	7	2	Sulphur....	59.6	61.0	63.5	68.7	69.3	53	179	126	—
Rat no 4.....	8	2	Gypsum....	55.0	56.8	57.5	58.7	55.8	53	157	104	—
3 Restricted feeding: <sup>1</sup>												
Rat no 1.....	1	0	None.....	36.5	62.4	72.2	72.4	-----	47	212	165	—
Rat no 2.....	2	2	do.....	36.2	64.6	78.7	73.3	-----	54	204	150	+
Rat no 3.....	3	2	Sulphur....	36.5	62.4	78.8	(?)	-----	51	(?)	-----	—
Rat no 4.....	2	2	Gypsum....	36.5	62.4	65.0	65.4	-----	51	205	154	—
Rat no 5.....	4	0	None.....	47.9	80.3	87.0	90.9	-----	40	258	218	—

<sup>1</sup> Feeding of rats 1, 3, and 4 restricted to that of no. 2.<sup>2</sup> Owing to exhaustion of the "selenium-plus-sulphur" grain sample, this rat was killed and autopsied a week before the others, at which time its weight was the same as that of the control on normal grain, i. e., 206 g.

During the first week of the unrestricted feeding experiments the rats on the grain samples from plots 2 and 6, treated with selenium alone, ate much less and grew far more slowly than those fed grain from the similarly selenized plots that had received sulphur or gypsum in addition (fig. 1, A and B). After the first week the difference became less pronounced as the rats apparently became used to the abnormal smell or taste of the "selenium-alone" grain and ate more normally, with a corresponding increase in rate of growth. Since marked initial retardation of growth is characteristic of animals given food containing selenium (5, 6, 8), the comparative growth rates during the first week of feeding showed clearly that the sulphur and gypsum treatments of the soil had so reduced the selenium intake of the plants that the grain did not retard the growth of the rats.

In the third experiment the growth rates of the rats on diets containing grain from plots 1, 3, and 4, respectively, each restricted in amount to that eaten by the rat on grain from plot 2, were all much the same (fig. 2), indicating that the initial retardation in the growth of the rats on grain from the unsulphured selenized plots 2 and 6 was largely attributable to disinclination to eat rather than to a specific physiological effect of the ingested selenium. Since the rat on the grain from plot 2 was fed all that he would eat, comparison of his growth curve with that of the fifth male of the same litter on an unrestricted diet of normal grain shows the extent to which the former's growth was hindered (fig. 2). The 2 p. p. m. selenium added to the soil did not make the grain sufficiently toxic to kill the rats in any of the experiments.

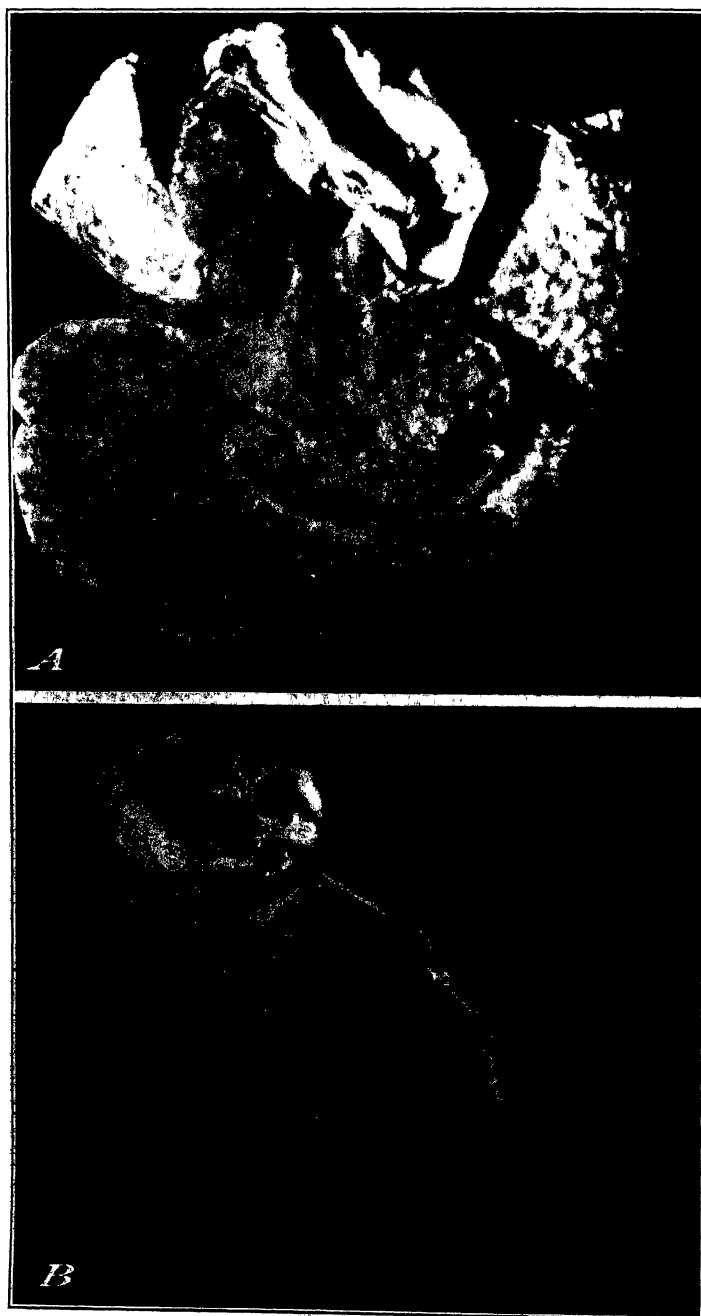


FIGURE 3.—*A*, Liver of rat of second experiment injured by selenium in grain grown on Keyport clay loam to which was added 2 p. p. m. of selenium as sodium selenate; *B*, normal liver.  $\times 2$ .

The evidence from the growth curves that the grain from the selenized soil treated with sulphur or gypsum was less toxic than that from the plots receiving selenium alone was corroborated by the autopsy findings. Liver injury is the most obvious effect of mild selenium poisoning in rats (5, 6, 8). In the present experiments such injury characterized all the rats on grain from the unsulphured selenized soils. The upper lobe was small and atrophied; the one immediately beneath was enlarged and thickened (fig. 3). In one case the lobes were held together by adhesions. The surfaces were roughened by prominent lobules, which produced a granular appearance rather than the extreme nodulose distortion accompanying more severe poisoning (5). In a photograph made with light from a mercury lamp (fig. 4, *C*) the liver had a blanched appearance suggestive of the anemic condition characteristic of selenized rats (5, 7, 12). In marked contrast, the livers of the rats fed grain from the selenized soil treated with sulphur or gypsum appeared normal (fig. 4, *D* and *E*), like those of the rats fed control grain from the unselenized plots (fig. 4, *A* and *B*). The livers therefore showed very convincingly that the excess sulphur added to the selenized soil had prevented the grain from absorbing toxic concentrations of selenium.

On analysis, the grain that produced the selenium-damaged liver shown in figure 4, *C*, was found to contain about 12 p. p. m. of selenium (making a concentration of 8.4 p. p. m. in the diet). The nontoxic grain from the similarly selenized plots given sulphur or gypsum applications contained about 4 p. p. m. (making 2.8 p. p. m. in the diet).

TABLE 2.—*Analyses of plant material from the plots producing the grain of the feeding tests*

Soil plot no.	Soil treatment		Effect of first year's grain on rats	Selenium in plants and grain--							
	Selenium added	Form of sulphur added		First year				Second year, grain <sup>3</sup>			
				Young plants <sup>1</sup>	Mature plants <sup>2</sup>	Grain <sup>3</sup>					
	<i>P. p. m.</i>			<i>P p m</i>	<i>P p m</i>	<i>P p m</i>	<i>P p m</i>	<i>P p m</i>	<i>P. p. m.</i>		
1	0	None.....	Nontoxic.....	0	0.5	-----	-----	0.0	0.0	0.0	
2	1	do.....	Toxic.....	110	12.0	-----	-----	2.0	1.0	1.0	
3	2	Sulphur.....	Nontoxic.....	2	3.0	3	-----	3	-----	1	
4	2	Gypsum.....	do.....	8	3.0	5	4	.1	-----	.2	
5	0	None.....	do.....	0	-----	-----	-----	-----	-----	-----	
6	2	do.....	Toxic.....	50	13.0	12	11	5.0	-----	6.0	
7	2	Sulphur.....	Nontoxic.....	2	7.0	4	3	.4	-----	.3	
8	2	Gypsum.....	do.....	5	3.0	4	4	.6	-----	.5	

<sup>1</sup> Gravimetric determinations by A. Van Kleeck under the direction of H. G. Byers, of the Bureau of Chemistry and Soils.

<sup>2</sup> Gravimetric determinations by R. F. Gardiner and R. B. Deemer under the direction of P. R. Dawson, of the Bureau of Plant Industry.

<sup>3</sup> Colorimetric determinations in duplicate by L. H. Greathouse, of the Bureau of Plant Industry

The analyses of grain from the various selenized plots are summarized in table 2 (except that from plot 2, which was entirely consumed in the feeding tests), together with analyses of the plants themselves at an early stage of development (tillering) and at maturity. Analyses of grain from the same plots the following year (1935) with no additional treatments of the soil are also included. The data all give evidence of reduced selenium absorption in the presence of

excess sulphur, the reduction being even more pronounced the second year than the first.<sup>5</sup>

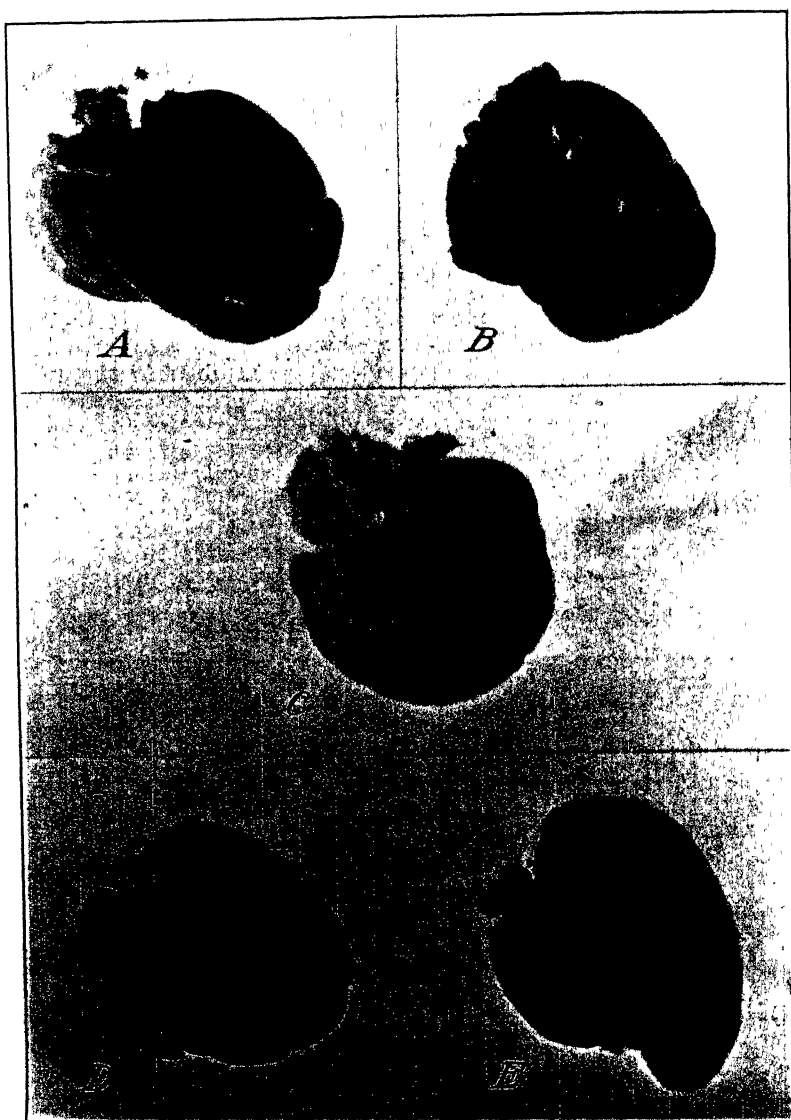


FIGURE 4—Livers of rats fed: (A and B) Control grain from plot 5; (C) grain from plot 6 to which 2 p. p. m. of selenium as sodium selenate was added; (D) grain from plot 8 receiving the same quantity of selenium plus gypsum; (E) grain from plot 7 receiving the selenium plus sulphur. All five livers were photographed on one plate with light from a mercury lamp. Natural size.

<sup>5</sup> A third year's analyses of young plants from these plots showed that but a trace of available selenium remained in the soil. The plants from the selenized plots 2 and 6 without sulphur or gypsum still contained the most selenium, namely, 0.6 and 0.7 p. p. m.—where 56 to 116 p. p. m., had been found 2 years before. Evidence of a rapid decrease in selenium absorption by successive crops on the same soil appears also in the grain analyses for the first and second years.



That grain grown on soil treated with but 2 p. p. m. of selenium (5 g of sodium selenate in 1,200 pounds of soil) should be toxic emphasizes the seriousness of the problem in areas where the soil contains even higher concentrations (3). The problem exists because of the capacity of the plant for accumulating large quantities of selenium from small amounts in the soil, a capacity possessed in extraordinary degree by certain forage plants of the Middle West where the selenium disease of livestock occurs (1, 2, 3, 4, 9). Whether sulphur treatments will prove fully effective and feasible for reducing the absorption of selenium by vegetation in these areas remains to be determined.

#### SUMMARY

In the present experiments wheat grown on soil containing 2 p. p. m. of selenium and comprising 70 percent of the diet of white rats produced the retarded growth and the liver injury characteristic of selenium poisoning. Wheat grown on similarly selenized soil treated with flowers of sulphur or with gypsum was not toxic. Chemical analysis of the grain showed that the sulphur and gypsum treatments reduced the concentration of selenium in the grain from about 12 p. p. m. to about 4 p. p. m.

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# RESPONSE OF CERTAIN PLANTS TO LENGTH OF DAY AND TEMPERATURE UNDER CONTROLLED CONDITIONS<sup>1</sup>

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## INTRODUCTION

Since the appearance of the first paper of Garner and Allard on photoperiodism (5)<sup>2</sup> an extensive literature covering various phases of the subject has developed. The accumulated data as a whole are sufficiently comprehensive and consistent to remove any doubt as to the practical importance of length of day as a factor in plant growth and development. However, only in comparatively few instances have the experiments in this field been conducted with sufficiently accurate control of the environment as a whole to yield results of a quantitative nature that could be readily reproduced. Since in most instances natural illumination, with or without supplemental artificial light, has been employed, control of the illumination conditions other than daily duration has been out of the question, and usually only partial control of other environmental factors has been attempted.

Inasmuch as growth and development within the limits imposed by heredity ordinarily are the net result of interaction of the many environmental factors influencing the plant, it is necessary to supplement the above-mentioned data with observations made under controlled conditions and with suitable modifications of factors other than light duration if accurate information is to be obtained on the relationship of other factors to the day-length effect. It is true that rigid control of the environment necessarily will introduce conditions differing more or less from those obtaining in nature, but insofar as normal growth and development of the plant can be obtained, this method appears to furnish the best available means for studying the interrelation of the light period and other factors.

In the present paper results obtained in growing typical short-day and long-day plants under controlled conditions are presented. The primary objectives were (1) to compare plant response to the daily light period under rigidly fixed environments involving artificial illumination with results previously obtained under more or less natural conditions and (2) to make a direct study of the interrelation of day length and temperature as ecological factors. To culture the higher plants under adequately controlled conditions offers many experimental difficulties and at best is both laborious and expensive, so the data presented are necessarily limited. These data, however, appear to throw additional light on the photoperiodic responses of the plants studied and on the relation of temperature to these responses. With the artificial climates employed it has been possible to obtain photoperiodic responses closely agreeing with those observed under outdoor conditions.

<sup>1</sup> Received for publication Jan. 23, 1936; issued July 1936.

<sup>2</sup> Reference is made by number (*italic*) to Literature Cited, p. 959.

## MATERIAL AND METHODS

Soybeans (*Soja max* (L.) Piper), sugar beets (*Beta vulgaris* L.), and *Rudbeckia bicolor* Nutt. were used in the experiments, all of which were concerned mainly with initiation of sexual reproduction. Special attention was given to the comparative responses of the Mandarin, Peking, and Biloxi varieties of soybean, which in the latitude of Washington, D. C., behave as early, medium, and late sorts, respectively. The seed used were of strains supplied by the Division of Forage Crops and Diseases. At Washington the Biloxi and Peking varieties are markedly delayed in flowering by exposure to the maximum day length of summer, amounting to nearly 15 hours, and clearly are to be classed as short-day types. Although the 15-hour day has only a very slight delaying action on time of flowering of the Mandarin, it will be shown in the present paper that this variety differs essentially from the earlier ones in its photoperiodic response only in that its critical light period—that is, the maximum day length at which flowering ordinarily takes place—is considerably in excess of the 15-hour maximum length of day at Washington. There appears to be no reason, therefore, why this plant should not be regarded as a short-day type (4). The beet seed used were of a selected strain furnished by the Division of Sugar Plant Investigations. Commercial seed of *Rudbeckia* were employed. The beet and *Rudbeckia* are long-day types.

The apparatus used for control of environment has been described by Steinberg (10). It consists essentially of eight small light-proof cabinets through which conditioned air is circulated at a definite rate and which are provided with lighting units designed to furnish high-intensity illumination from 1,000-watt gas-filled tungsten lamps without material disturbance of the temperature of the air about the plants. In the experiments with soybeans the old type of lighting equipment was used, consisting of the ordinary pear-shaped 1,000-watt lamp with metal reflector and a 3-inch screen of water flowing through a pan with a clear glass bottom placed between the lamp and the plants. In the tests with *Rudbeckia* and sugar beets the improved lighting unit, affording better horizontal distribution of light intensity, was used. This unit consists primarily of a special type of cylindrical lamp that burns immersed in distilled water contained in a clear glass globe, the distilled water being cooled by means of tap water flowing through a copper coil immersed in its upper layer.

At 12 inches below the light source the average illumination was about 2,600 foot-candles with either type of lighting equipment. At 28 inches below the light source the illumination was about 1,200 foot-candles, and it decreased but little at greater distances below the light source. The air velocity in the cabinets was maintained at about 0.9 mile per hour. Computations indicate that at this rate of air flow the concentration of the carbon dioxide could not be appreciably modified by the action of the plants. When the temperature level was varied the relative humidity also was changed in such a way as to maintain a constant saturation deficit.

The wooden containers used for growing the soybeans were made watertight by coating them on the inside with asphalt and were fitted with metal covers containing 2-inch holes through which the plants grew. Split perforated corks were used to close the openings

and hold the plants in place. Aluminum containers asphalted on the inside were employed in growing *Rudbeckia* and sugar beets. Covers could not be used because of the growth characteristics of these plants.

Two potting-soil mixtures composed of a heavy loam, manure, and a small component of sand were used. The first mixture was employed in all the experiments with soybeans except the one run at 89° F. During the tests the moisture content of this mixture was maintained automatically at approximately 17.4 percent. The moisture content of the second soil mixture, employed in all the remaining experiments, was similarly maintained at 24 percent. In each instance the soil moisture was approximately 54 percent of saturation, and the weight of the moist soil in each container was approximately 80 pounds.

In the experiments with soybeans 5 plants of each variety, or a total of 15 individuals, were grown in each container. In the experiment with sugar beets at 65° F. 9 individuals were grown in each container, but in the remaining tests the number of plants was reduced to 4 in order to avoid crowding. In all the *Rudbeckia* tests 9 individuals were allowed to develop in each container.

## EXPERIMENTAL DATA

### SOYBEANS

In the six experiments with soybeans comprising the principal series to be reported in detail, the daily light periods ranged from 3 to 17 hours and the four temperature levels employed were 71°, 77°, 83°, and 89° F. In order to make a more detailed study of the response to day length at 77°, the interval between light periods was only 1 hour, and since only eight periods could be used in a single test, two separate experiments were required to cover the range from 3 to 17 hours. The first of these embraced light periods ranging from 3 to 10 hours, inclusive, and the second embraced periods ranging from 10 to 17 hours, inclusive. The experimental data presented in table 1 for the light periods ranging from 3 to 10 hours and a temperature of 77° represent the average results of duplicate tests. Three experiments covering the longer light periods were carried out, but in two of these the control of temperature and humidity was not entirely satisfactory because of abnormal outdoor conditions, so the data reported in the principal series cover only a single experiment. At temperatures other than 77° the interval between light periods was 2 hours, so that in each instance a single experiment sufficed to cover the entire range in light periods. At each temperature level the relative humidity employed was that corresponding to a saturation deficit of 0.424 mm of mercury. The duration of the experiments ranged from 40 to 60 days.

The essential conditions applying to the individual experiments are as follows:

Experiment 1: Duration, 60 days or 1,440 hours; temperature,  $71^{\circ} \pm 0.5^{\circ}$  F.; relative humidity,  $44 \pm 1$  percent; light periods, 3 to 17 hours.

Experiment 2: Duration, 40 days or 960 hours; temperature,  $77^{\circ} \pm 0.5^{\circ}$ ; relative humidity,  $54.2 \pm 1$  percent; light periods, 3 to 10 hours.

Experiment 3: Duration, 44 days or 1,056 hours; temperature,  $77^{\circ} \pm 0.5^{\circ}$ ; relative humidity,  $54.2 \pm 1$  percent; light periods, 3 to 10 hours.

Experiment 4: Duration, 50 days or 1,200 hours; temperature,  $77^{\circ} \pm 1^{\circ}$ , except that for a period of 4 hours there was a mean departure of approximately  $+2^{\circ}$ ; relative humidity,  $54.2 \pm 2$  percent, except that for a total period of 50 hours there was a mean departure of about  $+4$  percent; light periods, 10 to 17 hours.

Experiment 5: Duration, 59 days or 1,416 hours; temperature,  $83^{\circ} \pm 0.5^{\circ}$ ; relative humidity,  $61 \pm 1$  percent; light periods, 3 to 17 hours.

Experiment 6: Duration, 44 days or 1,056 hours; temperature,  $89^{\circ} \pm 1^{\circ}$ ; relative humidity,  $69 \pm 2$  percent; light periods, 3 to 17 hours.

In these tests, as well as in those described later, the temperature and humidity levels and the range of departure from these levels

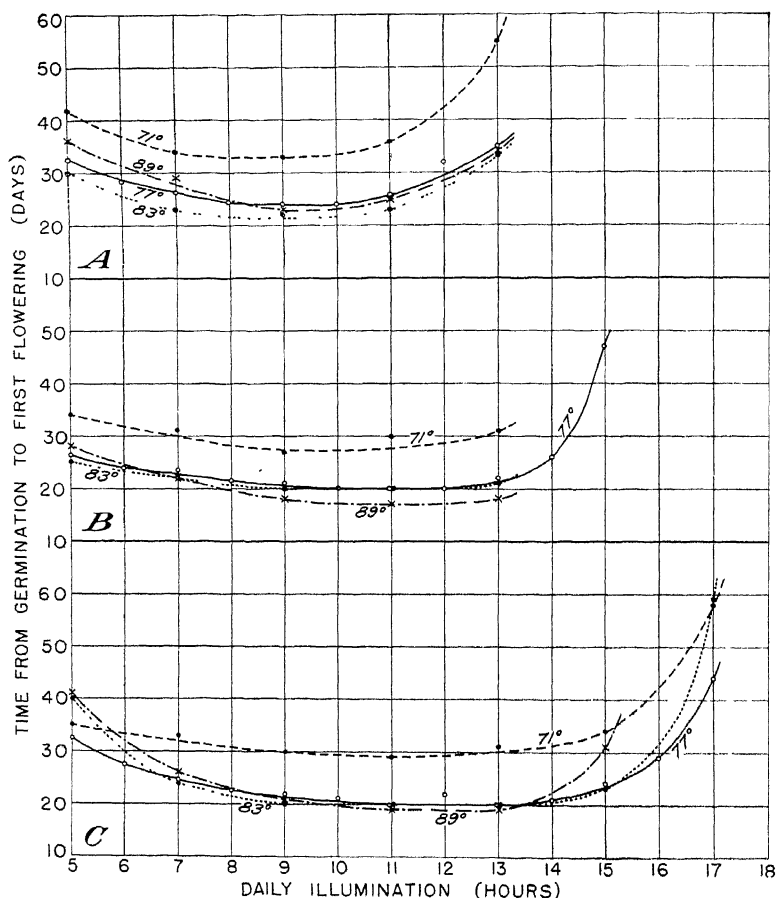


FIGURE 1.—Effect of daily light period on number of days elapsing from germination to appearance of first blossoms in Biloxi (A), Peking (B), and Mandarin (C) varieties of soybeans grown at various fixed temperatures.

pertain in all cases to the air as it entered the individual light cases. The possible disturbing effects of the lamps within the cases on the air temperature and humidity have been considered by Steinberg (10). Numerous observations within the cases have indicated that the temperature range incident to the going on and off of the lamps does not exceed  $\pm 0.5^{\circ}$  F.

In the above-described experiments observations were made on the number of days elapsing from germination to appearance of first blossoms and from germination to the average time of flowering of all individuals in each treatment, on the height of the plants at time of flowering and at the end of the test, and on the number of nodes and length of internodes of the plants. An attempt was made to obtain also the fresh and dry weights of the plants at the end of the experiments, but the data were rejected as unreliable primarily because of heavy shedding of the foliage leaves. The data bearing on duration of the vegetative stage and on growth characteristics are summarized in table 1 and figure 1. Unfortunately height measurements at time of flowering were not obtained in the experiments conducted at a temperature of 77° F., but the final heights of the plants were measured.

TABLE 1.—*Effect of daily light period on average number of days elapsing from germination to first flowering, on height of plants at flowering and at harvest, on number of nodes produced, and on average length of internodes in the Biloxi, Peking, and Mandarin varieties of soybeans grown at various fixed temperatures*

Temperature (° F.)	Daily illumination	Average time from germination to first flowering			Height of plants at—						Nodes produced			Average length of internodes		
					Flowering			Harvest								
		Biloxi	Peking	Mandarin	Biloxi	Peking	Mandarin	Biloxi	Peking	Mandarin	Biloxi	Peking	Mandarin	Biloxi	Peking	Mandarin
71	Hours	Days	Days	Days	Cm	Cm	Cm	Cm	Cm	Cm	Number	Number	Number	Cm	Cm	Cm
	5	51.8	34.6	41.0	42.9	28.5	28.1	46.2	32.9	31.4	6.4	5.6	4.8	7.2	5.9	6.9
	7	35.4	34.3	34.8	35.7	20.5	25.1	54.1	25.4	27.8	8.0	6.0	5.6	6.8	4.2	5.0
	9	33.6	30.0	31.4	41.0	24.0	26.2	81.8	37.6	34.1	10.8	7.0	6.6	7.6	5.4	5.2
	11	38.4	31.6	31.0	44.5	20.9	25.8	84.1	37.7	38.1	12.4	6.6	7.2	6.8	5.7	5.3
	13	55.6	32.6	33.4	88.3	26.3	26.7	105.4	46.0	45.3	16.6	7.5	7.6	6.4	6.1	6.0
	15	---	---	---	---	---	---	100.7	88.9	85.7	16.0	13.5	12.2	6.3	6.6	7.0
	17	---	---	---	---	---	---	106.2	102.2	107.0	16.8	15.0	15.8	6.4	7.1	6.8
	77	4	43.0	33.5	---	---	---	106.2	28.1	34.8	4.7	4.0	4.0	10.3	7.0	8.7
	6	35.3	27.3	35.0	---	---	---	60.8	34.9	38.4	6.4	5.5	4.5	9.5	6.4	8.5
8	29.4	25.0	28.8	---	---	---	65.9	37.5	41.4	7.2	6.0	4.9	9.1	6.3	8.4	
7	27.3	23.8	25.8	---	---	---	67.1	40.3	44.6	7.2	5.6	5.3	9.3	7.2	8.4	
8	26.0	22.8	23.5	---	---	---	77.2	42.8	45.6	8.6	6.0	5.9	9.0	7.1	7.7	
9	25.0	22.3	23.0	---	---	---	76.9	43.4	51.2	9.8	5.9	6.1	7.9	7.4	8.4	
10	26.7	21.2	22.5	---	---	---	94.0	52.8	64.2	10.4	6.0	7.0	9.0	8.8	9.2	
11	27.0	21.6	22.4	---	---	---	92.5	56.1	62.9	7.8	6.0	7.6	11.9	9.4	8.3	
12	32.7	20.8	24.4	---	---	---	117.3	62.8	84.6	10.0	6.2	8.3	11.7	10.1	10.2	
13	36.3	22.2	22.0	---	---	---	130.5	74.5	84.6	13.7	8.4	8.8	9.5	8.9	9.6	
14	---	27.0	22.0	---	---	---	80.0	90.3	80.4	8.3	9.8	8.5	9.7	9.8	9.5	
15	---	---	25.2	---	---	---	91.3	141.0	122.7	9.5	14.8	10.0	9.6	9.5	12.3	
16	---	---	32.0	---	---	---	113.0	128.5	141.6	12.5	17.0	14.2	9.0	7.6	10.0	
17	---	---	45.0	---	---	---	109.1	123.0	121.8	11.2	13.5	13.3	9.7	9.1	9.2	
83	5	39.4	25.6	41.5	50.4	33.0	36.5	59.6	46.2	39.4	7.2	7.0	5.3	8.1	6.6	7.4
	7	27.0	23.2	25.3	51.0	31.6	43.8	100.2	49.7	54.6	11.6	7.8	6.0	8.7	6.4	9.1
	9	23.2	21.0	21.4	51.9	30.5	45.7	154.6	40.3	91.6	17.0	7.2	8.4	9.1	6.8	10.9
	11	25.2	21.0	21.4	61.6	31.8	40.3	126.3	46.5	72.8	17.6	6.8	8.4	7.2	6.8	8.7
	13	41.0	22.8	20.6	120.5	39.0	51.5	165.0	52.1	91.7	22.4	7.8	9.0	7.4	6.7	10.2
	15	---	---	25.6	---	---	---	143.0	136.4	179.0	23.0	15.0	15.2	6.2	9.1	11.8
	17	---	---	59.7	---	---	186.8	184.0	139.9	183.4	24.3	14.4	16.2	6.7	9.7	11.3
89	5	36.0	29.6	41.0	81.5	42.8	49.0	76.8	45.7	50.4	7.0	6.4	5.6	11.0	7.1	9.0
	7	33.6	24.2	28.0	95.4	40.4	66.3	125.3	58.3	71.8	11.0	7.8	5.6	11.4	7.5	12.8
	9	25.0	18.4	26.2	68.2	39.3	74.3	129.0	69.7	102.8	13.6	7.9	5.4	9.5	8.9	19.0
	11	27.2	17.6	20.2	88.5	48.1	84.1	145.7	82.5	93.6	13.2	8.4	7.0	11.1	9.8	13.4
	13	34.0	20.6	19.6	93.5	63.3	100.1	144.5	101.3	184.5	16.4	13.6	7.0	8.8	12.4	18.2
	15	---	---	34.3	---	---	127.5	158.0	170.3	186.3	15.6	13.6	11.2	10.0	12.5	16.6
	17	---	---	---	---	---	---	159.7	168.2	192.0	17.0	13.8	10.6	9.4	12.1	18.1

In view of the fact that the tray screen used in the soybean tests was less efficient with respect to horizontal distribution of illumination than the globular screen utilized in the experiments with *Rudbeckia*

and beet, a special experiment was made to compare the two types of screen in their effects on flowering in soybeans exposed to certain light periods. The essential conditions of the test were: Duration, 39 days or 936 hours; temperature,  $77^{\circ} \pm 0.5^{\circ}$  F.; relative humidity,  $54.2 \pm 1$  percent; light periods, 5, 10, and 15 hours. The results are shown in table 2.

Ability to reproduce results obtained under given conditions perhaps constitutes one of the best means of testing the effectiveness of the control measures employed. The data on initiation of flowering obtained in experiments 2 and 3, the conditions of which already have been outlined, throw light on this phase of the investigation. The results are shown in table 3.

TABLE 2.—Comparative effects of tray and globular types of water screen placed between light source and test plants on number of days elapsing from germination to appearance of first blossoms in Biloxi, Peking, and Mandarin soybeans exposed to various light periods and a temperature of  $77^{\circ} \pm 0.5^{\circ}$  F.

Daily illumination (hours)	Biloxi		Peking		Mandarin	
	Tray screen	Globular screen	Tray screen	Globular screen	Tray screen	Globular screen
5.....	Days 35	Days 29	Days 26	Days 25	Days 32	Days 31
10.....	25	25	20	21	20	20
15.....					24	24

TABLE 3.—Results of duplicate experiments, A and B, to determine effect of daily light period on number of days elapsing from germination to appearance of first blossoms in Biloxi, Peking, and Mandarin soybeans grown under controlled conditions at a temperature of  $77^{\circ} \pm 0.5^{\circ}$  F.

Daily illumination (hours)	Biloxi		Peking		Mandarin	
	A	B	A	B	A	B
5.....	Days 32	Days 33	Days 26	Days 27	Days 33	Days 32
6.....	28	29	24	24	27	28
7.....	26	27	23	22	24	25
8.....	24	25	22	22	22	23
9.....	23	25	21	21	21	23
10.....	23	25	20	21	21	21

The illumination conditions obtained with the gas-filled tungsten lamp and water screens used in the present experiments differ decidedly from those of natural illumination, especially as to the composition of the light and the daily curve of illumination intensity. For this reason it is of considerable importance to determine whether the photoperiodic responses obtained with the artificial illumination are in line with those previously observed with sunlight. In table 4 comparison is made between the results obtained with a 10-hour daily illumination period in the present tests at  $77^{\circ}$  F. and those obtained at comparable temperatures with 10 hours of sunlight in outdoor summer plantings and with the full day of the winter months in greenhouse plantings. Similar agreement in effects of the artificial and natural illumination was obtained with other daily light periods.



TABLE 4.—*Comparison of natural and artificial illumination as affecting duration of the vegetative period of Biloxi, Peking, and Mandarin soybeans under short-day conditions*

Conditions of test	Mean temperature	Biloxi	Peking	Mandarin
Outdoor plantings at 1-day intervals through June and July, 10-hour day	° F. 75 1-80 4	Days 23 6	Days 19 2	Days 19 4
Greenhouse plantings at 5-day intervals in December, January, and February, natural day of 9 5-12 hours	75-79	26 8	21 5	23 1
Controlled conditions, tungsten filament lamp, 10-hour day	77±0.5	24 0	20 0	21. 0

## SUGAR BEETS AND RUDBECKIA

The experiments with sugar beets were carried out at three temperature levels, and in each instance light periods of 6, 9, 12, 13, 14, 15, and 18 hours and continuous light were employed. In the first experiment, which was run at 65° F., a saturation deficit of 0.25 mm of mercury was maintained. It was found, however, that this saturation deficit would be excessive for the higher temperature used in the second test, since no artificial refrigeration was available. Consequently in the later tests, covering both the highest and lowest temperatures used, the saturation deficit was maintained at 0.183 mm. The essential conditions applying to the three experiments were as follows:

Experiment 1: Duration, 77 days or 1,848 hours; temperature, 65°±1° F.; relative humidity, 59±1 percent, except that for a total period of 52 hours there was an average departure of -0.4 percent.

Experiment 2: Duration, 100 days or 2,400 hours; temperature, 60°±1°, except that for an aggregate period of 100 hours there was an average departure of -1.8°; relative humidity, 65±1 percent.

Experiment 3: Duration, 56 days or 1,344 hours; temperature, 73°±0.5°; relative humidity, 76.5±1 percent. Although it was not possible to maintain accurate control of temperature and humidity beyond the period of 56 days, the cultures were continued under rough control for an additional period of 3 weeks to determine whether flowering would occur under conditions other than continuous light. In this interval the temperature range was 73° to 78° and that of the relative humidity 76.5 to 83 percent. However, there was no further flowering.

The significant data on flowering in the three experiments are given in table 5. For the duration of the tests there was no reproductive response under the light periods shorter than 18 hours.

TABLE 5.—*Effect of daily light period on number of days elapsing from germination to appearance of first blossoms in sugar beets grown at 60°, 65°, and 73° F.*

Daily illumination (hours)	60° F.	65° F.	73° F.
	Days ( <sup>1</sup> )	Days ( <sup>1</sup> )	Days ( <sup>1</sup> )
15			
18	65	66	
24	65	48	39

<sup>1</sup> Plants did not flower.

Only two experiments were conducted with *Rudbeckia*. The light periods used were the same as for the sugar beets. As in the tests with soybeans, the relative humidity was that corresponding to a saturation deficit of 0.424 mm of mercury.

Experiment 1: Duration, 60 days or 1,440 hours; temperature,  $83^{\circ} \pm 0.5^{\circ}$  F; relative humidity,  $61.2 \pm 1$  percent.

Experiment 2: Duration, 54 days or 1,296 hours; temperature,  $77^{\circ} \pm 1^{\circ}$ , except that there was an average departure of  $+1^{\circ}$  for a total period of 8 hours; relative humidity,  $54.2 \pm 1$  percent, except that for a total period of 84 hours there was an average departure of  $+4$  percent.

The results obtained in the two experiments are shown in table 6. In the experiments at  $77^{\circ}$  F. stem elongation occurred with the 13-hour day, but the plants had not flowered when the experiments were terminated. There was no flowering with day lengths of 6, 9, or 12 hours at either temperature.

TABLE 6.—Effect of daily light period on number of days elapsing from germination to appearance of first blossoms and from germination to average time of flowering of all individuals and average height attained by the stem of *Rudbeckia bicolor* grown at  $77^{\circ}$  and at  $83^{\circ}$  F.

Daily illumination (hours)	Time elapsing from germination to first flowering at—		Time elapsing from germination to average date of flowering of all individuals at—		Average height of plants at time of—			
					Flowering at—		Harvest at—	
	77° F.	83° F.	77° F.	83° F.	77° F.	83° F.	77° F.	83° F.
	Days	Days	Days	Days	Cm	Cm	Cm	Cm
13.....		55		56 5		62	22	52
14.....	44	38	47	45 0	46	62	55	83
15.....	49	34	51	36 5	81	66	77	98
18.....	35	29	41	32 0	56	66	84	102
24.....	35	29	43	31 0	64	61	87	106

## INTERPRETATION AND DISCUSSION OF RESULTS

For the most part the experimental material was grown under closely controlled conditions, and the data for soybeans given in table 3 show that with the equipment and methods employed it is possible to satisfactorily reproduce experimental results. That the growth and development of the experimental material obtained in the artificial climates employed closely approximate results under natural conditions is shown in figure 2. Although the improved lighting assembly with the globular water screen possesses several advantages over the older lighting system with the tray type of water screen, the latter also can be made to yield satisfactory results (table 2). It is a matter of special interest that with the artificial climates used in the present experiments the number of days elapsing from germination to appearance of first blossoms in the three varieties of soybean in response to favorable daily light periods agrees closely with results obtained with plantings grown with natural illumination under comparable temperature conditions (table 4) and previously reported in part (7). In this connection it may be stated that observed differences of 1 or 2 days in the time elapsing from germination to first flowering are not ordinarily significant, since it becomes largely a matter of individual judgment as to whether opening of a particular blossom is to be regarded as occurring on a given day or on the following day.

The number of days from germination to first flowering in each variety of soybean as affected by day length at each of the four temperature levels employed is shown graphically in figure 1. The corre-

sponding data for the average number of days required for all individuals in each test to reach the flowering stage are given in table 1. In view of the small number of plants used, the results as a whole are remarkably consistent. At each of the temperatures employed a selective or differentiating action of day length on the three different varieties with respect to the critical light period is clearly in evidence. With favorable temperature conditions the critical light period for the Mandarin lies in the region of a 17-hour day, that of the Peking around a 15-hour day, and that of the Biloxi between a 13- and 14-hour day. Additional observations with narrower differences in day length

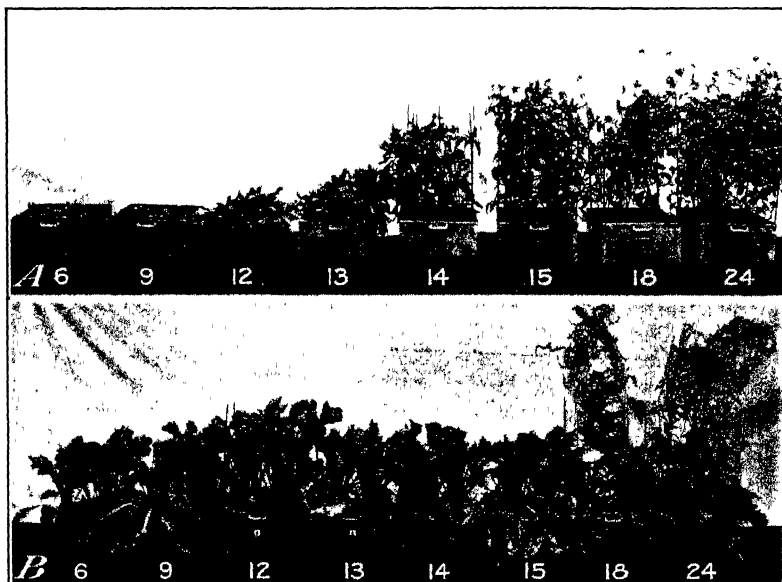


FIGURE 2—*Rudbeckia bicolor* (A) and sugar beets (B) grown with various daily light periods ranging from 6 hours to continuous light, as indicated by numbers below the plant containers, and at fixed temperatures of 77° and 60° F., respectively. These cultures illustrate the type of growth and development obtained with the artificial climates employed.

are needed to fix more accurately these critical light periods for the particular conditions involved. As the temperature becomes increasingly unfavorable the critical light period tends to become somewhat shorter, as shown, for example, in the result with Mandarin at 89° F. and that with Biloxi at 71°. For each variety there is a rather wide range in day lengths that are shorter than the critical light period and are approximately optimum for flowering. For the Mandarin this range is about 9 to 14 hours, for the Peking 8 to 12 or 13 hours, and for the Biloxi 7 to 11 hours, although distinctly unfavorable temperatures tend to narrow somewhat these ranges. Perhaps the true optimum day-length ranges can be somewhat more narrowly defined as the zones lying between 11 and 13 hours for the Mandarin, 9 and 12 hours for the Peking, and 8 and 10 hours for the Biloxi.

It is of interest to consider the relative efficiency of the light energy in initiating reproductive activities under conditions of constant intensity and composition but with varying daily duration of light. Taking

for this purpose the product of the daily hours of light and the number of days elapsing from germination to appearance of first blossoms, it appears that, as shown graphically in figure 3, exclusive of the general region of the critical light period, the values for each variety and each temperature employed tend to form ascending linear series. The total number of light hours elapsing from germination to first flowering increases with increase in length of the daily light period, maximum efficiency of the light being attained with the shortest day length which will maintain the plant. Hence, graphs representing

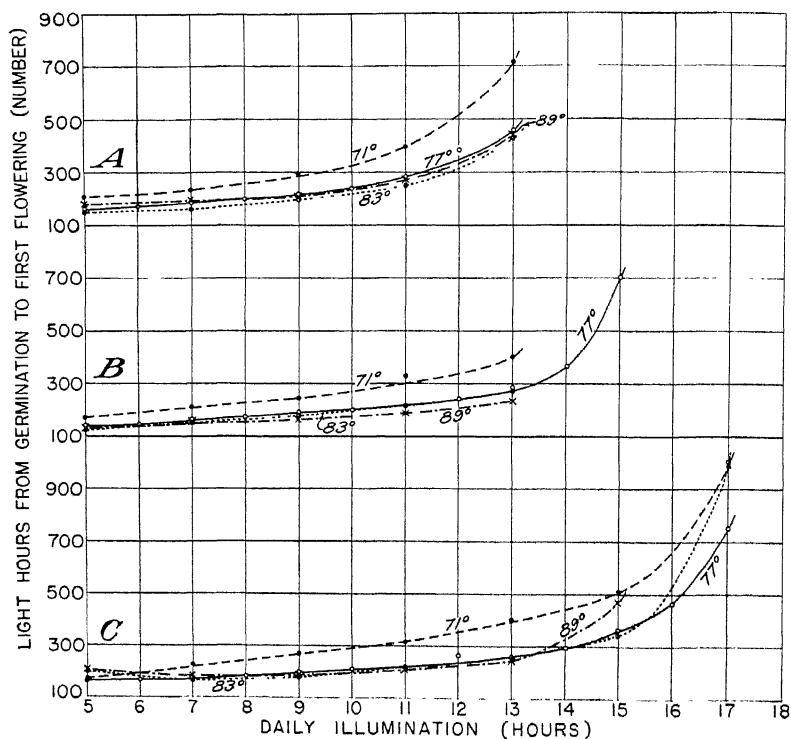


FIGURE 3.—Effect of daily light period on total number of light hours elapsing from germination to appearance of first blossoms in the Biloxi (A), Peking (B), and Mandarin (C) varieties of soybeans grown at various fixed temperatures.

this relationship differ in form from those of figure 1 except in the region of the critical light period, where they assume the same form. It seems probable that the increase in number of days elapsing from germination to first flowering with the shortest light periods employed as compared with the intermediate light periods (fig. 1) is due to conditions of impaired nutrition rather than to any weakening of the formative action of the light-darkness ratio. None of the soybean varieties was able to survive with a 3-hour daily period of light, while the Biloxi and Peking flowered with a 4-hour period and the Mandarin flowered with a 5-hour period.

As always has been the case when the soybeans were grown with natural illumination, the factor of heredity in relation to relative

number of days elapsing from germination to appearance of first blossoms in the different varieties under optimum conditions comes into play in the present data, although its effect is small. As shown by the values in figure 1 and the corresponding average values in table 1, the minimum number of days elapsing from germination to first flowering in the Biloxi exceeds the minimum in the Mandarin by about 3 days, and the minimum in the latter exceeds that in the Peking by 1 or 2 days.

The effect of temperature on the duration of the vegetative stage is clearly evident in the results obtained (table 1 and fig. 1). The lowest temperature employed ( $71^{\circ}$  F.) was distinctly suboptimum, and flowering was delayed in all varieties and with all day lengths. The next higher temperature ( $77^{\circ}$ ) is much nearer the optimum for all varieties and all day lengths. The third temperature level ( $83^{\circ}$ ) gave practically the same results with Mandarin and Peking as were obtained at  $77^{\circ}$  for all day lengths except in the region of the critical light period, but  $83^{\circ}$  appears to be nearer the optimum than  $77^{\circ}$  for the Biloxi. The highest temperature employed ( $89^{\circ}$ ) was somewhat excessive on the whole, especially for the Biloxi, although at optimum day lengths this temperature slightly accelerated flowering in the Peking and Mandarin as compared with lower temperatures.

The interrelation of temperature and day length as affecting soybeans has been studied by Eaton (2) and by Gilbert (8). By subjecting Peking soybeans that were growing in a 13-hour day to a night temperature of about  $50^{\circ}$  F., Eaton obtained a considerable delay in flowering in comparison with similar plants exposed to night temperatures of  $65^{\circ}$  and  $90^{\circ}$ , respectively. Gilbert made observations on Mandarin, Peking, and Biloxi soybeans grown under short-day conditions in the greenhouse, one lot of each being exposed to a mean temperature of  $65^{\circ}$  to  $70^{\circ}$  and a relative humidity of 85 percent, while corresponding material received a mean temperature of  $75^{\circ}$  to  $80^{\circ}$  and a relative humidity of 50 percent. Under the latter conditions all varieties showed flower buds in about 30 days, while at the end of this period there was no indication of flowering at the lower temperature and higher humidity.

It is apparent that the soybean is decidedly a warmth-loving type. In the data herein presented there is no evidence of a definite selective or differentiating effect of temperature on the three varieties as to time elapsing from germination to appearance of first blossoms. Apparently the effects of temperature could not explain the fact that when planted in early summer in the latitude of Washington, D. C., the Mandarin, Peking, and Biloxi invariably behave as early, medium, and late-maturing varieties, respectively. The falling temperature in late summer or fall would tend to retard rather than hasten flowering in the late variety, while the relatively high temperature of mid-summer would favor early flowering in this variety. In line with previous findings (7) it appears that length of day rather than temperature is primarily responsible for the observed differences in the three varieties with respect to time of flowering and fruiting when grown in moderately high latitudes. However, it seems clear that a relatively high mean temperature is required for success in all of these varieties.

The data in table 1 afford a basis for computing temperature coefficients for flowering of the Biloxi, Peking, and Mandarin soybeans

through a range in temperature of 18° F. at various day lengths. These coefficients are shown in table 7. It is evident that all are below the values (2-3) ordinarily associated with chemical reactions, and progressively increase in magnitude with increase in day length. If the size of the temperature coefficient be indicative of the type of reaction, as is usually assumed, it would seem that the reactions that are associated with flowering in the plant differ from those applying to vegetative growth. With increasing day length the purely chemical reaction appears to play an increasingly greater part in determining the time of the flowering process, as indicated by the increasing values of the temperature coefficients. It is felt that the divergencies in 2 of the 16 values do not vitiate these conclusions inasmuch as the remaining data are relatively uniform even though based on only two experiments, and, moreover, the variations occur near the region of greatest error, namely, the critical day length for flowering. Again the uniformity in values for the three varieties at each of the day lengths tested indicates that flowering is influenced by temperature to approximately the same degree in all these varieties, and that their differentiation in nature into late, medium, and early varieties, respectively, cannot be dependent on temperature.

TABLE 7—*Temperature coefficients of flowering in soybeans at various day lengths for a range of 18° F.*

Daily illumination (hours)	Biloxi			Peking			Mandarin		
	Time elapsing from germination to flowering at—		Coeff- icient	Time elapsing from germination to flowering at—		Coeff- icient	Time elapsing from germination to flowering at—		Coeff- icient
	71° F.	89° F.		71° F.	89° F.		71° F.	89° F.	
	Days	Days		Days	Days		Days	Days	
5.....	42	36	1.17	34	28	1.21	35	41	1.1
7.....	34	29	1.17	31	22	1.41	33	26	1.27
9.....	33	23	1.43	27	18	1.50	30	21	1.43
11.....	36	25	1.44	30	17	1.76	29	19	1.63
13.....	55	34	1.62	31	18	1.72	31	19	1.63
15.....							34	31	1.10

<sup>1</sup> Equals 0.85, and indicates a delay rather than a hastening in the time of flowering.

Because of the small number of individuals grown in each test, conclusions to be drawn from the data on growth relations necessarily must be somewhat tentative. As a result of crowding and other factors, individuals that obtained a poor start often tended to lag in growth; so the average values do not present smooth series such as were obtained in the data on flowering. Whether the plants under the various treatments attained the flowering stage, and also the time required to reach this stage, would be expected, of course, to modify the rate and especially the ultimate amount of growth. At 89° F. the attained height of the Mandarin at time of flowering increased progressively with increase in the daily number of hours of light (table 1), and apparently attenuation was associated with this response. In no other instance was there definite increase in attained height of the plants at flowering time in response to increase in the daily light period except in the general region of the critical

light period. Within the latter range of day lengths the height increased rapidly with increase in the light period. For reasons which are not clear these results differ somewhat from those obtained by various investigators working with natural illumination. Under the latter conditions increase in the light period through the lower ranges usually has resulted in increased height of the plants at time of flowering. However, the average daily rate of growth during the pre-flowering stage showed a clearly defined tendency to increase with increase in the photoperiod in all of the varieties at 83° and 89°. At 71° temperature apparently was the principal limiting factor, and only the Biloxi showed appreciable increase in growth rate in response to increase in day length. With respect to effects of temperature, with only a single exception, each increment increased both the attained height at flowering and the average daily gain in height for each day length employed.

The final height at time of harvest on the whole tended to increase with increase in the duration of the daily light period. In the Mandarin and Peking the rate of increase with increase in the light period was much more marked as the critical light period was approached, whereas in the Biloxi the height increased more or less uniformly with increase in duration of the light period. Interpretation of the data bearing on the relation of temperature to final height of the plants is complicated somewhat by the fact that the tests conducted at different temperatures were not continued for the same number of days. The average duration of all tests was approximately 50 days. When the data on final height in table 1 are adjusted on this basis, it appears that with all varieties and all day lengths employed increase of temperature from 71° to 77° F. materially increased the height of the plants. Further increase in temperature from 77° to 83° produced comparatively little effect. The final temperature increase from 83° to 89°, however, produced a further marked increase in plant height, this effect increasing somewhat with increase in day length. Even the unadjusted data of table 1 show on the whole a decided increase in height of the plants with increase in temperature.

The effects of varying the daily period of light on the number of nodes produced by the individual plant were similar to the effects on final height of the plants. In the Biloxi the number of nodes tended to increase more or less uniformly with increase in day length, whereas in the Mandarin and Peking the relative increase in number of nodes produced with increasing day length was much more marked in the region of the critical light period. The effects of temperature on the number of nodes produced and the average length of internodes become more clearly defined when the observed data are adjusted to a basis of 50 days as the duration of each test, as was done in considering temperature effects on the final heights of the plants. On the whole, the number of nodes, especially in the Biloxi, tended to increase with the increase in temperature, and in the case of the other two varieties with the increase from 71° to 77° F. At the higher temperatures employed, especially at 89°, increase in the photoperiod produced a pronounced increase in the average length of internodes in the Peking and Mandarin, but in other cases showed little or no consistent effect. Increase in temperature from 71° to 77° produced a definite increase in length of internodes in each variety and at all

day lengths, and there was a further increase in internode length, mainly in the Mandarin, at 89°, the highest temperature employed. On the whole, length of internode was least affected by temperature in the Biloxi and most affected in the Mandarin.

The data obtained with sugar beets are too limited to afford a comprehensive picture of the interrelationship of temperature and duration of illumination as affecting reproductive activity, but they throw considerable light on the subject. The beet is recognized as being a pronounced long-day type, and it has been found by several investigators also that in this plant stem elongation, flowering, and seed formation are favored by relatively low temperature. In the present tests flowering occurred in 65 days with an 18-hour day and with continuous illumination at a temperature of 60° F., but with increase of temperature the 18-hour light period progressively decreased in effectiveness. With continuous light, however, the time elapsing from germination to appearance of first blossoms decreased with increase in temperature up to 73°. It does not follow, of course, that maximum amount of flowering and fruiting would occur at the higher temperature. Under the conditions and for the duration of the tests there was no stem elongation or flowering with light periods shorter than 18 hours. In all cases there was considerable enlargement of the root.

In earlier experiments in the greenhouse, conducted by Garner in collaboration with Allard, the results of which have not been published in detail though briefly referred to in a nontechnical paper (3), several varieties of table beet as well as sugar beet developed seed-stalks and flowered freely without material thickening of the root when exposed to a 16- to 17-hour day and a mean temperature of about 55° F. The plants received the full day of winter supplemented with high-intensity artificial light till midnight, and the daily range in temperature was about 10°. The time required for flowering was 130 to 135 days from germination. With similar illumination and a mean temperature of approximately 72° no stem elongation or flowering occurred, but there was decided enlargement of the root and the leaves were quite long. Under the short-day conditions there was no stem elongation at either temperature during the period of the tests, while there was more pronounced thickening of the root at the higher than at the lower temperature. In a prior experiment with the table beet (6) large roots taken from winter storage and grown under outdoor conditions of temperature and full day length in late spring soon flowered. Similar plants exposed to a 10-hour day produced short seedstalks, but the apical buds subsequently developed into leaf rosettes and there was no flowering.

With a day length of 14.5 to 15 hours and a mean temperature of 65° F. or slightly lower, Skuderna<sup>3</sup> obtained flowering within 75 days in sugar beet seedlings growing in the greenhouse. Supplementing daylight with high-intensity electric light for the entire night and avoiding the high temperatures of midsummer, Munerati (9) was able to grow five generations of beets in 1 year, obtaining mature seed in 52 to 66 days after planting. With a temperature of 50° to 66°, Chroboczek (1) obtained elongation of seedstalks but no flowering in table beets exposed to an 8-hour day, and sugar beets failed to produce

<sup>3</sup> SKUDERNA, A. W. PHOTOPERIODISM. Amer. Beet Sugar Co., Research Dept. 1926. [Unpublished.]



seedstalks under the short-day conditions. With continuous illumination flowering occurred, even at a temperature of 70° to 80°.

It seems clear that the beet, which is a long-day type, differs also from the soybean, a short-day type, in that the temperature range favorable to reproductive activity in the former is considerably lower than that favoring reproduction in the latter. On the other hand, it appears that within the favorable range of temperature the time elapsing from germination to appearance of first blossoms in the beet tends to decrease with increase of temperature in much the same way as in the soybean. In either case change in temperature may modify to some extent the response to length of day, and vice versa, although it does not necessarily follow that the mode of action of temperature and of day length is the same. Apparently the beet does not require a winter rest period and commonly behaves as a biennial only because in most situations it is grown under combined conditions of light and temperature that are more or less unfavorable for reproductive activity.

The data in table 6 indicate that the critical light period for *Rudbeckia* is 13 hours or somewhat shorter, at least for the temperatures employed. Beyond this point increase in the day length up to 18 hours decreases the time elapsing from germination to appearance of first blossoms, but the 18-hour day is as effective as continuous light. The final height of the plants increases with increase in day length up to 18 hours. Increase in temperature from 77° to 83° F. decreases the time elapsing from germination to first flowering and increases the final height of the plants. *Rudbeckia*, a long-day type, resembles the beet in its response to length of day, while it rather closely resembles soybeans in its temperature requirements. As in the case of both the beet and the soybean, within the range of temperature favorable to reproductive activity the time elapsing from germination to first flowering decreases with increase of temperature.

From the standpoint of the interrelationship of length of day and temperature as environmental factors, soybeans, sugar beets, and *Rudbeckia* may be regarded as representing three somewhat contrasted types with respect to reproductive response. For the beet, a combination of long day (or continuous light) and cool temperature affords optimum conditions; for *Rudbeckia bicolor*, a combination of long day and warm temperature; and for late-maturing (Biloxi) soybeans, a combination of short day and warm temperature. The early-maturing (Mandarin) soybean differs essentially from the late-maturing variety only in that it has a considerably longer critical light period, and in this respect the medium variety (Peking) occupies an intermediate position. The fourth possible type of plant, in which reproductive activity is favored by a short day combined with cool temperature, is not represented in the present experiments. The work of Gilbert (8) indicates that *Cosmos* is a representative of this type.

During the open growing season of temperate regions the mean temperature tends to decrease with increase in latitude, although locally such factors as altitude may modify this tendency. There is definite increase in length of day, of course, with increase in latitude. Accordingly, in most situations the beet would be more likely to behave as an annual at very high latitudes. For *Rudbeckia bicolor* the favorable effect on reproductive activity of the long days of very high latitudes might be offset by the lower mean temperature. In high

latitudes the lower temperature would be unfavorable alike to the early and late varieties of soybeans, but the latter, because of their shorter critical light period, would be at a distinct disadvantage as compared with the former. Plants of the type to which *Cosmos* belongs probably are better adapted to relatively high latitudes than is the group represented by the late-maturing variety of soybeans, for although the two groups have similar day-length requirements the falling temperature of late summer or early fall is more favorable for the former.

#### SUMMARY

Fairly extensive observations were made on response to length of day of early-, medium-, and late-maturing varieties of soybeans grown at mean temperatures of 71°, 77°, 83°, and 89° F. under controlled conditions. Limited observations also were made on sugar beets grown at mean temperatures of 60°, 65°, and 73°, and on *Rudbeckia bicolor* Nutt. grown at mean temperatures of 77° and 83°. The plants were grown with high-intensity illumination from gas-filled tungsten-filament lamps in specially constructed chambers, and various day lengths were used.

With the artificial climates employed the reproductive response of soybeans to length of day agreed closely with results previously obtained with natural illumination under comparable conditions. With the artificial climates it was found possible to satisfactorily duplicate results.

At each of the temperatures employed there was definite contrast in the response of the three varieties of soybeans with respect to the critical day length for flowering, that is, the maximum length of day at which the plants tended to become reproductive. With favorable temperature conditions the critical light period for the early variety (Mandarin) was found to be in the region of a 17-hour day; that of the medium variety (Peking), around a 15-hour day; and that of the late variety (Biloxi), between a 13-hour and a 14-hour day. The optimum day length for flowering was not sharply defined, but that of the Mandarin seemed to range from approximately 11 to 13 hours, that of the Peking from 9 to 12 hours, and that of the Biloxi from 8 to 10 hours.

The soybean was found to have a relatively high temperature requirement, and there appeared to be only slight differences in the requirements of the three varieties used in the tests. The lowest mean temperature employed (71° F.) delayed flowering in each of the varieties at all day lengths. Temperature differences altered the critical light periods to a limited degree, but there were no marked contrasts in these effects on the three varieties. Response to length of day appears to be primarily responsible for the fact that the Mandarin, Peking, and Biloxi normally behave as early, medium, and late varieties, respectively.

The total number of light hours elapsing from germination to appearance of first blossoms increased with increase in length of the daily light period, maximum efficiency of the light being attained with the shortest day length capable of maintaining the plant. The efficiency of the light energy increased with increase of temperature.

Because of the small number of individuals grown in each test and their uneven growth rate, only tentative conclusions may be drawn from the data obtained on growth relations in the soybeans. At the

higher temperatures the average daily growth rate during the pre-flowering stage increased with increase in day length, but at 71° F. temperature appeared to be the chief limiting factor. The final height of the plants tended to increase with increase in the daily light period. The height at time of flowering as well as the final height tended to increase with increase in temperature. Increase in length of day increased the number of nodes produced, but did not greatly affect the length of the internodes except that in the Mandarin and Peking there was a marked increase at the higher temperatures. Increase in temperature also tended to increase both the number of nodes and their length, the latter effect being most obvious with the increase from 71° to 77°.

The sugar beet has been found to be a pronounced long-day type, and it also differs from soybeans in that the range of temperature favorable to reproductive activity is relatively low. The strain of sugar beet used in the tests flowered in 39 days when exposed to continuous light and a mean temperature of 73° F. As the daily light period decreased, lower temperature became increasingly favorable to flowering. Apparently the beet commonly behaves as a biennial rather than as an annual only because in most situations it is grown under combinations of day length and temperature that are more or less unfavorable to reproductive activity.

*Rudbeckia bicolor* resembles the beet in being a long-day type, while its temperature requirements are very similar to those of soybeans. The final height of the plants was found to increase with increase in day length up to 18 hours and with increase of temperature from 77° to 83° F.

From the standpoint of the interrelationship of length of day and temperature as environmental factors, soybeans, *Rudbeckia bicolor*, and beet represent three somewhat contrasted types with respect to reproductive activity. In late-maturing soybeans flowering and fruiting are favored by a combination of short day and warm temperature, in *Rudbeckia* by a combination of long day and warm temperature, and in the beet by a combination of long day and cool temperature. However, within the temperature range favorable to reproductive activity, whether this be warm or cool, in each instance increase in temperature hastens reproductive processes. The critical light period for flowering may be altered to a limited degree by temperature and, conversely, the favorable temperature range for flowering may be shifted by the action of day length.

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# SCRIBNER VOLUME TABLES FOR CUT-OVER STANDS OF PONDEROSA PINE IN ARIZONA<sup>1</sup>

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## INTRODUCTION

In connection with recent growth studies of cut-over stands of ponderosa pine (*Pinus ponderosa* Laws.) in Arizona, the Southwestern Forest and Range Experiment Station found it expedient first to construct new volume tables to fit the cut-over stands. The necessary measurements were made on an existing logging operation on felled timber similar in form to the residual stand. This was in line with Meyer's<sup>2</sup> findings in his analysis of data on ponderosa pine forests in the Pacific Northwest, that volume tables of this species may apply equally well to virgin stands or cut-over stands in that type, provided an adjustment is made in computing stand volumes where the cutting has greatly disturbed the balance of form classes.

On the basis of these data<sup>3</sup> it is the purpose of this paper to present (1) Scribner board-foot volume tables obtained from an equation (having only a small residual error<sup>4</sup>) adapted to the computing of volume increment in cut-over stands of ponderosa pine, and (2) a comparison of the "blackjack", "intermediate", and "yellow pine" forms of the species as to volume. These three terms, in common use locally, are employed to designate the three distinctive growth forms of ponderosa pine, namely, the very tapering immature trees with their dark, deeply furrowed, and narrow-plated bark; the transitional form with reduced taper and lighter bark; and the mature "yellow" pine, full-boled, with wide-plated, shallow-furrowed, yellowish-brown bark.

## CHARACTER OF DATA

The data used in this investigation were collected in 1932 from trees felled during lumbering operations of the Cady Lumber Corporation on five sections on the Sitgreaves National Forest, Ariz. The stand was pure ponderosa pine, considered typical of the volcanic formation of the Southwest known as the Malpais soil type. The height of mature dominant trees averaged 95 feet. The area is gently rolling with low flat-topped hills and wide shallow canyons. Its aspect is in general southwesterly, and the altitude is about 7,500 feet.

As indicated above, since the volume tables were to be applied to cut-over stands, measurements were made only on trees (selected at random) of a type approximating as nearly as possible that of the trees reserved in Forest Service cutting, i. e., normal, live-topped trees free from external defects. Care was taken to reject trees with

<sup>1</sup> Received for publication Mar. 2, 1936; issued July 1936.

<sup>2</sup> MEYER, W. H. EFFECT OF RELEASE UPON THE FORM AND VOLUME OF WESTERN YELLOW PINE. *Jour. Forestry* 29: 1127-1133, illus. 1931.

<sup>3</sup> Acknowledgment is due the staff of the Southwestern Forest and Range Experiment Station and the personnel of the Sitgreaves National Forest for help in collecting the field data.

<sup>4</sup> "Residual error" as used here refers to variations in volume that are not dependent upon variations in diameter and height.

defects (such as bad fire scars, dead or bayonet tops, forked tops, or severe lightning injury) that would result in abnormality of diameter, height, or bark thickness.

With mean ground level as the base, diameter and bark measurements were taken on each tree with adjusted calipers to the nearest 0.1 inch at heights of 1, 3, 4.5, and 10 feet, and at intervals of 8 feet above that, with, usually, a shorter interval at the tip. The value recorded in each case was the mean of two measurements taken at right angles. The diameters at breast height<sup>5</sup> were remeasured with a diameter tape as a check on the caliper measurements. Bark thickness was measured to the nearest 0.1 inch with a Swedish bark-measuring instrument; the figure recorded for bark thickness was the sum of two measurements taken at points diametrically opposite. Total height was measured to the nearest 0.1 foot with a steel tape.

The three-man crew collecting the field data were experienced both in Forest Service timber-marking practice and in ponderosa pine timber-sale work in Arizona, New Mexico, and Idaho. In classifying trees as blackjack, intermediate, or yellow pine, each tree was analyzed on the basis of character and color of bark, shape and condition of crown, and character of tip. In case of disagreement among the three men, the classification agreed on by two was accepted. Since age was found to be a fair criterion of classification, judgment on this point was checked by a count of annual rings on the stump. Diameter and height growth of ponderosa pine is normally slow; the range and mean age of the three groups was found to be: 40 to 165 years, with a mean of 109, for blackjacks; 110 to 250 years, with a mean of 176, for intermediates; and 180 to 410 years, with a mean of 270, for yellow pines.

#### ANALYSIS OF DATA<sup>6</sup>

Each tree for which data were plotted was scaled by the Scribner log rule in 16-foot saw logs with trimming allowance of 0.3 foot, a stump height of 1 foot, and a top diameter of 8 inches inside bark. Top sections less than one full saw log were scaled as fractions of a 16-foot 8-inch saw log. A multiple correlation<sup>7</sup> of logarithm of (volume—6.6) with logarithm of (diameter breast high inside bark—8 inches) and logarithm of (height—4.5 feet) was made for 119 blackjacks by methods described by Schumacher and Hall.<sup>8</sup> The calculated statistics are given in table 1.

TABLE 1.—Means and standard deviations of the logarithms of (diameter breast high inside bark, in inches—8), (total height, in feet—4.5), and (volume in board feet—6.6); and gross correlation between logarithms of the variables, for 119 blackjacks

Variable (X)	Mean (M)	Standard deviation (σ)	Correlation coefficient (r)
$X_1$ = Logarithm of (volume in board feet—6.6).....	Logarithm $M_1=1.9911$	Logarithm $\sigma_1=0.5160$	$r_{12}=+0.9557$ $r_{13}=+0.7289$ $r_{23}=+0.6199$
$X_2$ = Logarithm of (diameter breast high inside bark in inches—8).....	$M_2=.6215$	$\sigma_2=.3787$	
$X_3$ = Logarithm of (total height in feet—4.5).....	$M_3=1.7589$	$\sigma_3=.0990$	

<sup>5</sup> 4.5 feet above the ground; abbreviated d. b. h.

<sup>6</sup> Acknowledgement is due F. X. Schumacher and R. A. Chapman of the Forest Service for assistance and valuable suggestions in the statistical analysis.

<sup>7</sup> In which the origin of coordinates is placed at 8 inches d. b. h., 4.5 feet in total height, and 6.6 board feet in volume.

<sup>8</sup> SCHUMACHER, F. X., and HALL, F. DOS S. LOGARITHMIC EXPRESSION OF TIMBER-TREE VOLUME. Jour. Agr. Research 47: 719-734, illus. 1933.

The logarithmic regression equation for these statistics, computed according to the method given by Yule,<sup>9</sup> is

$$\log (\text{volume}-6.6)=b_{12.3} \log (\text{diameter breast high inside bark}-8)+b_{13.2} \log (\text{height}-4.5)+\log (\text{of the constant term}),$$

or

$$X_1=1.1151X_2+1.1555X_3-0.7343,$$

and the multiple correlation coefficient  $R_{1.23}=+0.9715$ . The coefficient of multiple correlation measures the combined importance

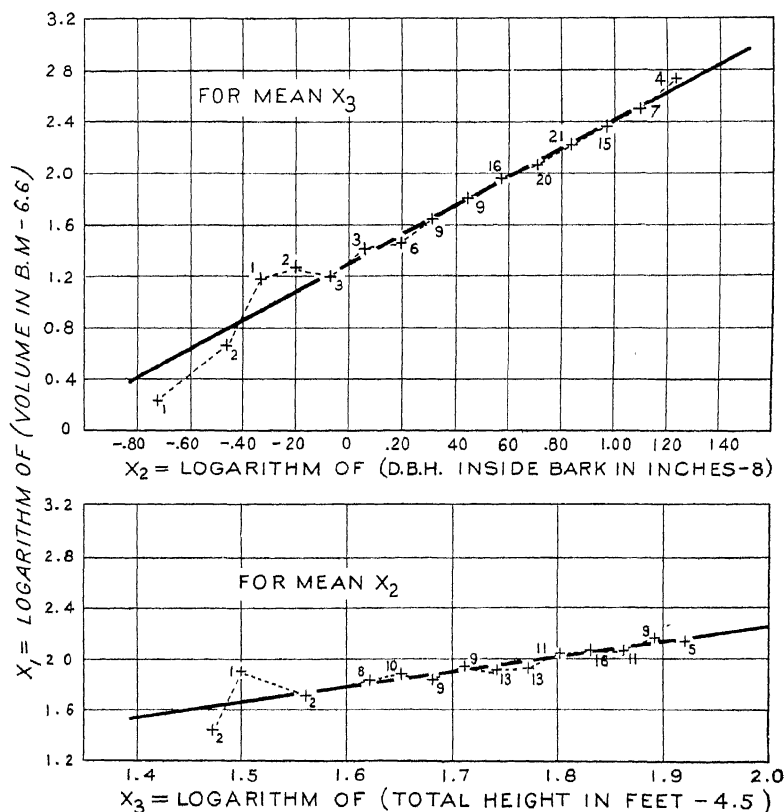


FIGURE 1.—Graphic test of the linearity of a logarithmic volume equation based on measurements of 119 blackjack trees.

of  $X_2$  and  $X_3$  as a means of explaining the differences in  $X_1$ , 0 and 1 equaling no correlation and perfect correlation, respectively.

The test of the linearity of the relationship of the logarithm of (volume - 6.6) to the logarithms of (diameter breast high inside bark - 8) and (height - 4.5) was made by comparing the actual  $X_1$  value of each tree with the estimated  $X_1$  value. The estimated  $X_1$  values were read from an alinement chart constructed from the volume equation. The results are presented in figure 1. The fit of the data to the calculated equation is very good.

<sup>9</sup> YULE, G. U. AN INTRODUCTION TO THE THEORY OF STATISTICS. Ed. 8, rev., 422 pp., illus. London. 1927. (See pp. 229-252.)

The same type of equation was fitted to the data of the intermediate and yellow pine groups and to the combined group of blackjacks and intermediates. The graphic tests for linearity are given in figures 2, 3, and 4, in which, as also in figure 1, the dependent variable is the logarithm of board-foot volume by the Scribner log rule to a constant top diameter of 8 inches, less 6.6 board feet. In every case the fit

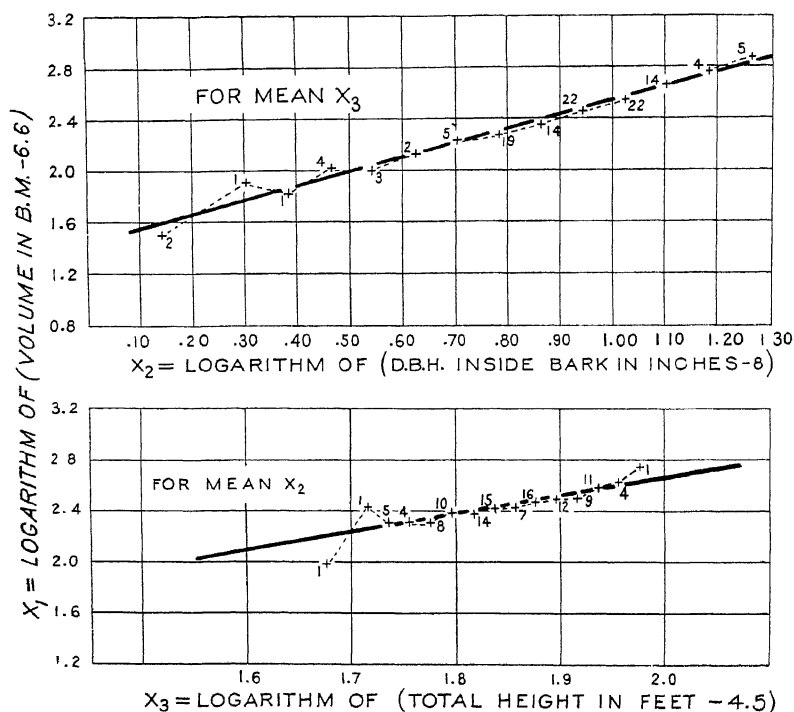


FIGURE 2.—Graphic test of the linearity of a logarithmic volume equation based on measurements of 118 intermediate trees.

of the data to a straight line is excellent and confirms the hypothesis presented by Schumacher and Hall.<sup>10 11</sup> The logarithmic regression coefficients, the constant term, and other statistics for the data of each of these groups are given in table 2.

<sup>10</sup> SCHUMACHER, F. X., and HALL, F. DOS S. See footnote 8.

<sup>11</sup> "Timber-tree volume equations of the power or logarithmic type in terms of diameter and height are deduced from known volume equations of geometrical solids on the theory that the volume of the tree stem, unlike that of the geometrical solids, may vary not directly as height and as the square of diameter, but as other powers of these dimensions in both cubic feet and board feet. The theory is tested by transforming the power equation into a linear one in logarithms and calculating the logarithmic regression equations for available tree data."



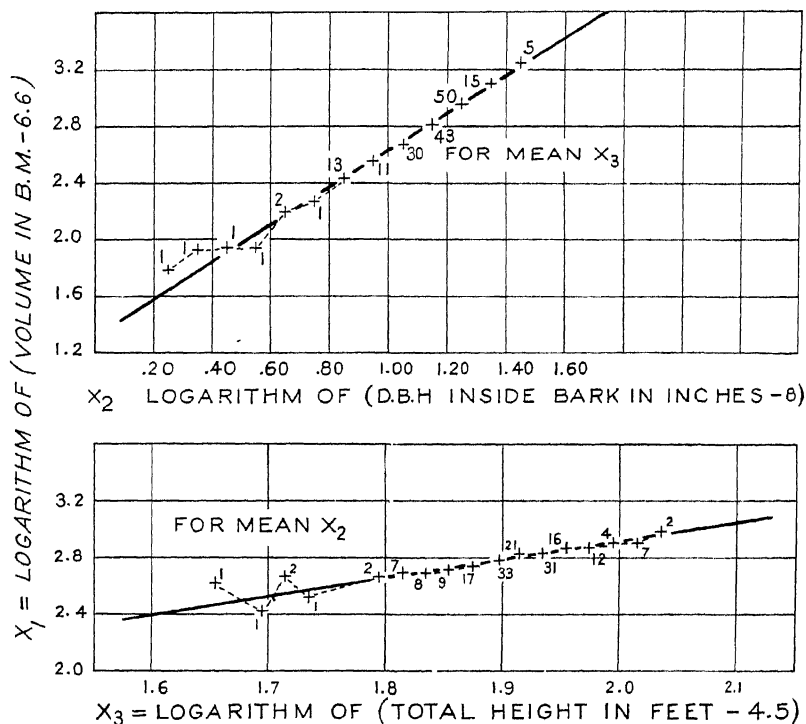


FIGURE 3.—Graphic test of the linearity of a logarithmic volume equation based on measurements of 174 yellow pine trees.

TABLE 2.—Logarithmic regression coefficients, constant terms, and other statistics for blackjacks, intermediates, and yellow pines, and blackjacks and intermediates combined, for each of which groups the logarithmic equations of Scribner board-foot volume were calculated

Class	Partial regression coefficients		Constant term	Means		
	$b_{12}$	$b_{13}$		$M_1$	$M_2$	$M_3$
Blackjack.....	1.1151	1.1555	-0.7343	Log 1.9911	0.6215	Log 1.7589
Intermediate.....	1.1068	1.3627	-1.0793	2.4363	.8996	1.8492
Yellow pine.....	1.3183	1.2943	-1.1479	2.8002	1.1220	1.9076
Combined blackjack and intermediate.....	1.1201	1.3329	-1.0596	2.2081	.7694	1.8050

Class	Standard deviations			Trees	Standard error of estimate $\sigma_{123}$	Gross correlation coefficient $r_{23}$	Multiple correlation coefficient $R_{123}$	Coefficient of determination $R^2_{123}$
	$\sigma_1$	$\sigma_2$	$\sigma_3$					
Blackjack.....	Log 0.5160	Log 0.3787	Log 0.0990	119	0.1224	+0.6199	0.9715	0.9437
Intermediate.....	.3169	.2154	.0623	118	.0863	+ .6544	.9622	.9259
Yellow pine.....	.3203	.1907	.0610	174	.0700	+ .7117	.9758	.9522
Combined blackjack and intermediate.....	.4837	.3371	.0938	237	.1032	+ .6895	.9770	.9545

The coefficient of determination,  $R^2_{1\ 23}$ , shows the percentage of variance in log (volume -6.6) that is associated with variance in log (diameter breast high inside bark -8) and log (height -4.5). The coefficient of determination for yellow pine is shown by table 2 to be 95.22 percent. Thus only 4.78 percent of the variance in log (volume -6.6) is due to factors other than log (diameter breast high inside bark -8) and log (height -4.5). Accordingly these factors were

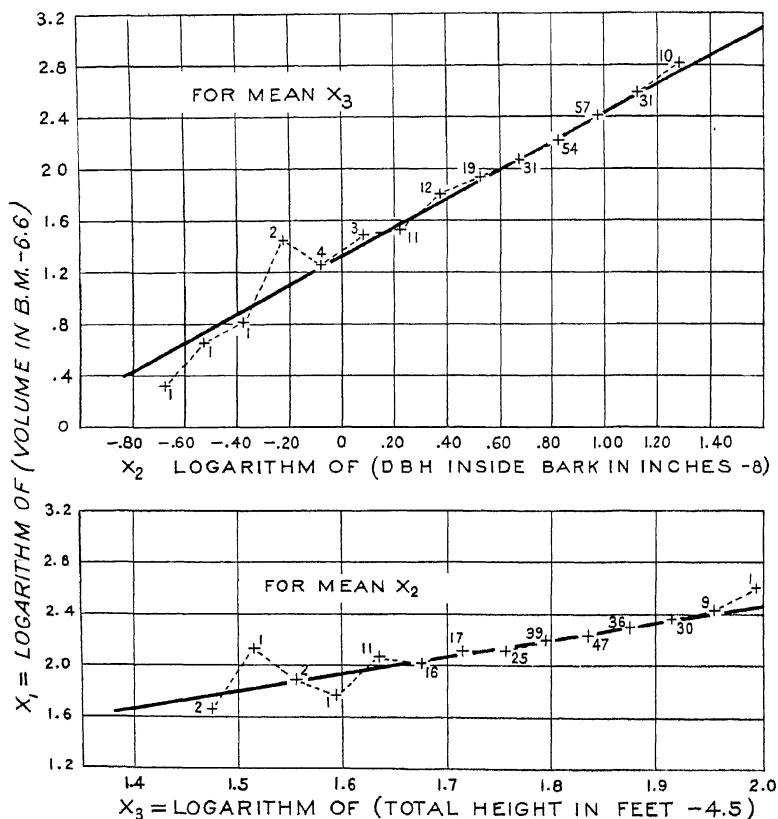


FIGURE 4.—Graphic test of the linearity of a logarithmic volume equation based on measurements of 237 blackjack and intermediate trees.

not considered in these computations. It should be noted, however, that the standard error of estimate,  $\sigma_{1\ 23}$ , is a measure of the accuracy with which the estimated log (volume -6.6) agrees with the actual log (volume -6.6) of the trees used in the study. From table 2 we find that the logarithmic volume equation for yellow pine is

$$X_1 = 1.3183 X_2 + 1.2943 X_3 - 1.1479,$$

and that the standard error of estimate is  $\pm 0.0700$ .

#### COMPARISON OF VOLUME EQUATIONS

Comparison of the volume equations of blackjacks, intermediates, and yellow pines resolves itself into a test of the significance of the difference in the log (volume -6.6) of individual trees of a given diam-

eter breast high and height, as estimated by the volume equation calculated for each group. This test was accomplished through the use of the standard error of the function, i. e., of the regression equation, as given by Miner<sup>12</sup> and as applied to volume-table tests by Schumacher and Hall.<sup>13</sup>

With the logarithmic equations as the functions and twice the standard error of the difference between values as calculated by two

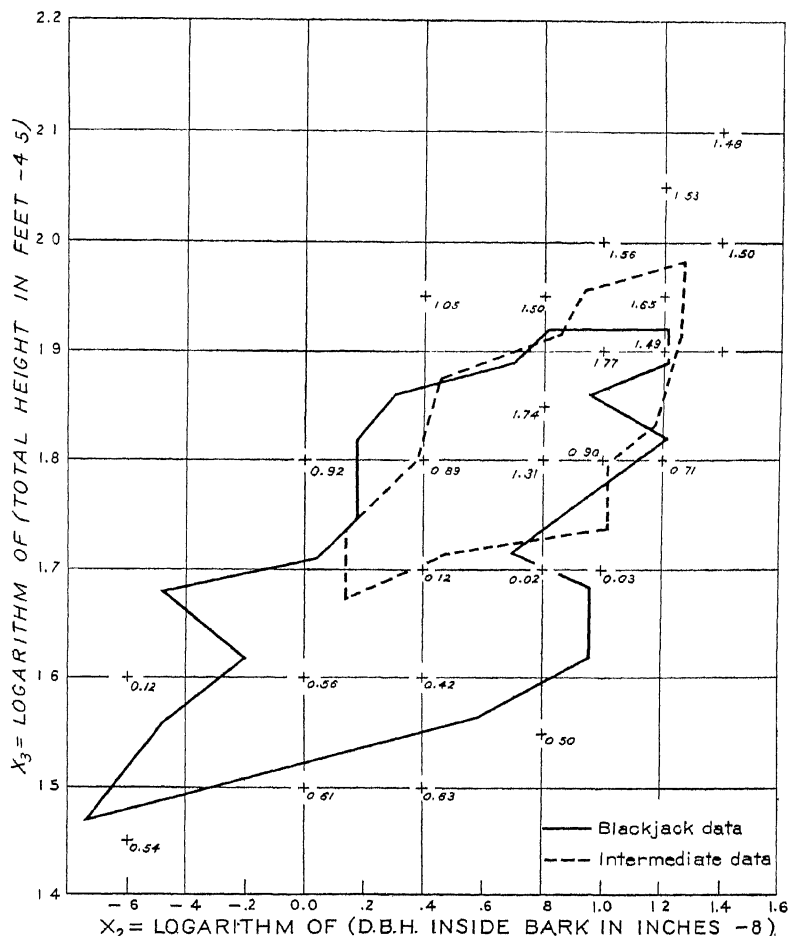


FIGURE 5.—Graphical representation of the test for determining the significance of the difference between the volume equation of the blackjack group and of the intermediate group. The volumes computed by the equations for these two groups are not significantly different

functions for trees of the same size as the lower limit of significance,<sup>14</sup> tests were made between blackjacks and intermediates, blackjacks and yellow pines, and intermediates and yellow pines. Graphic representations of the tests are given in figures 5, 6, and 7, in which

<sup>12</sup> MINER, J. R. THE STANDARD ERROR OF A MULTIPLE REGRESSION EQUATION. *Ann. Math. Statist.* 2: 320-323 1931.

<sup>13</sup> SCHUMACHER, F. X., and HALL, F. DOS S. See footnote 8.

<sup>14</sup> Roughly, the point at which the odds are 1 to 20 that the two samples came from the same universe.

each small cross (+) indicates the pairs of values of  $X_2$ ,  $X_3$  for which volumes were computed by each equation. The number near each cross is the ratio of the difference in volume, computed by each equation, to the standard error of the difference. A ratio of 1.9599 or larger is considered significant. It should be noted that the area

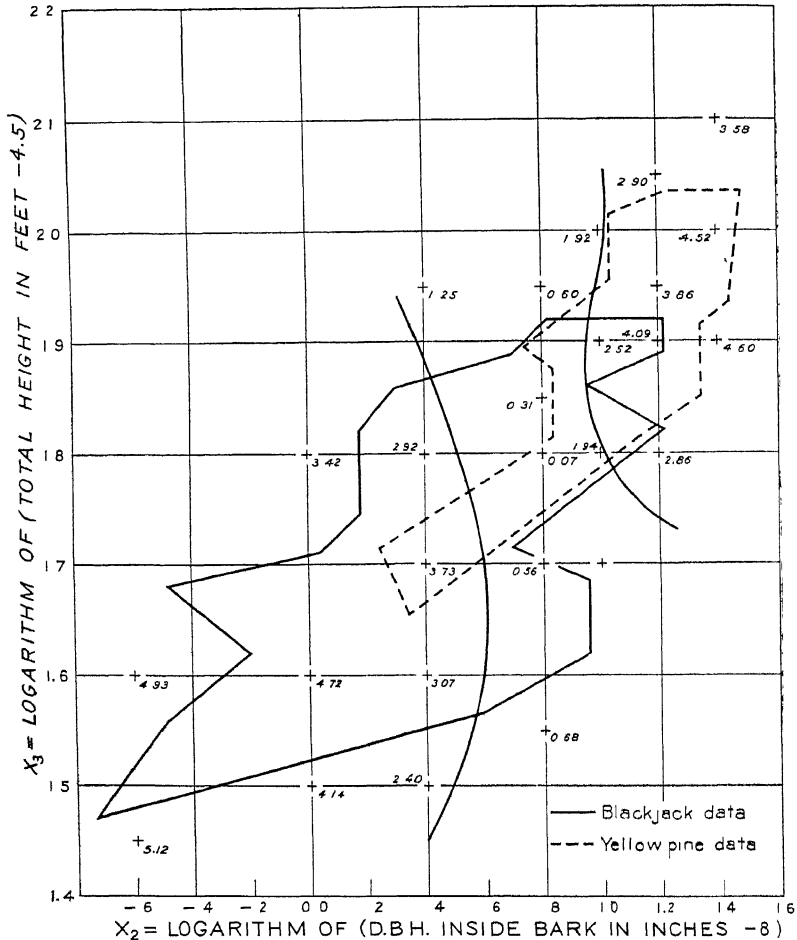


FIGURE 6.—Graphical representation of the test for determining the significance of the difference between the volume equation of the yellow pine group and of the blackjack group. The volumes, as estimated by the two equations, are significantly different excepting in the region of  $X_2$ ,  $X_3$ , lying between the two contours.

between the contours of two standard errors of the difference represents the distribution of logarithms of (diameter breast high  $-8$ ) and (height  $-4.5$ ) in which there is no significance or where either volume equation could be used satisfactorily.

Since there was no significant difference between the equations for blackjacks and intermediates, these groups were combined and

compared with yellow pines. Figure 8 is a graphic representation of this test and shows that the volume equations for blackjacks and intermediates combined is significantly different from that of yellow pines throughout the major part of their range of diameters and heights. Applying the data used in this test to figure 8, it was found

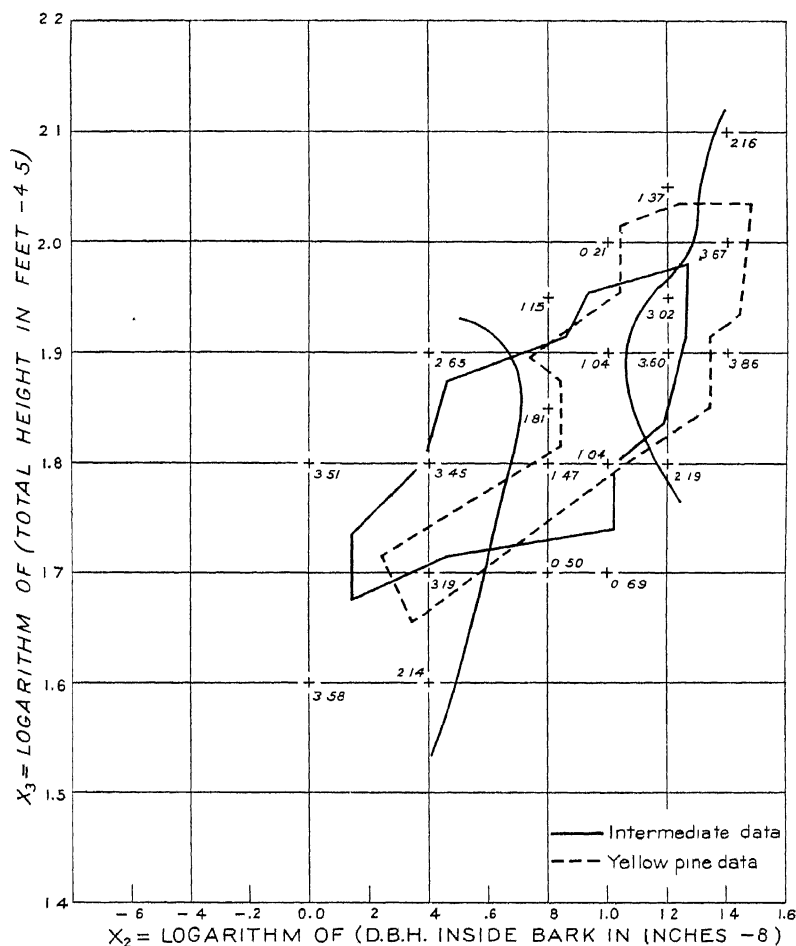


FIGURE 7.—Graphical representation of the test for determining the significance of the difference between the volume equation of the yellow pine group and of the intermediate group. The volumes, as estimated by the two equations, are significantly different excepting in the region of  $X_2$ ,  $X_3$  lying between the two contours.

that 10.9 percent of the yellow pines and 43.4 percent of the blackjacks and intermediates combined fell within the region of no significance or where either equation could be used satisfactorily. This means, so far as these data are concerned, that two volume tables should be constructed, one for yellow pines and one for the combined group of blackjacks and intermediates.

## ALINEMENT-CHART VOLUME TABLES

The alinement-chart form of volume table is especially valuable for use in growth or increment studies, since it makes it possible to use diameter measurements to the nearest one-tenth inch and height

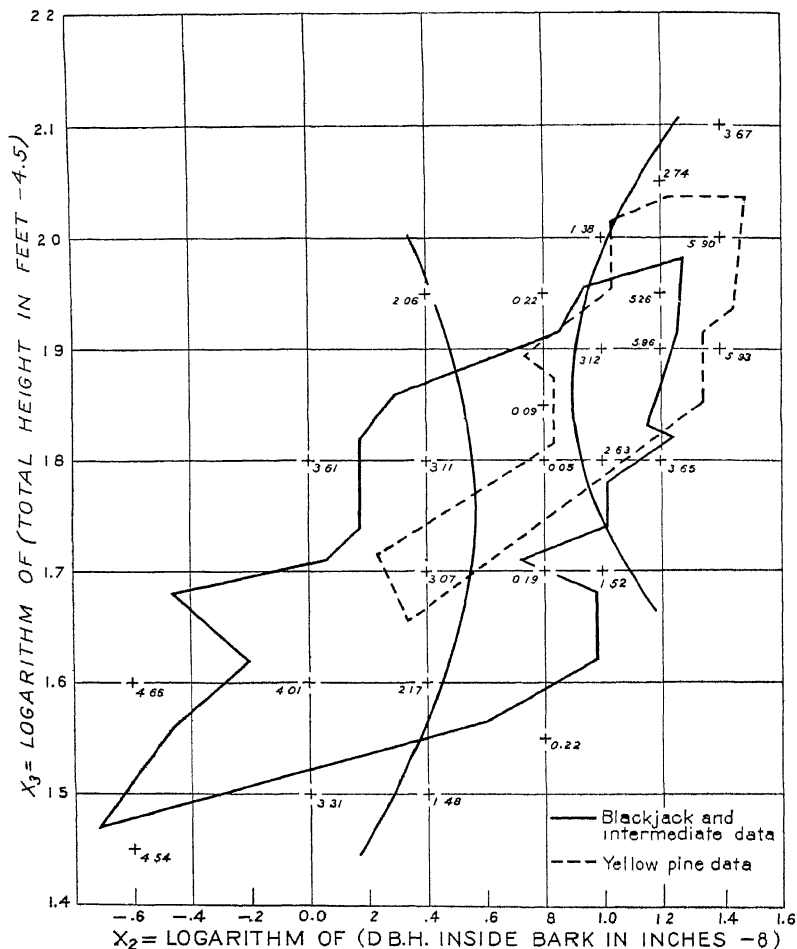


FIGURE 8.—Graphical representation of the test for determining the significance of the difference between the volume equation of the yellow pine group and of the combined blackjack-intermediate group. The volumes, as computed by the two equations, are significantly different excepting in the region of  $X_2$ ,  $X_3$ , lying between the two contours.

measurements to the nearest foot and to determine volumes of small and large trees with the same relative degree of accuracy.

The logarithmic form of the volume equations which have been presented is readily expressed in alinement-chart form. Three straight parallel axes for log (diameter breast high — 8), log (volume — 6.6), and log (height — 4.5), respectively, are used, upon which the logarithmic functions of the variables are graduated in the arith-

metric scale.<sup>15</sup> Corrections are made for constants that were subtracted from the variables before these were entered into logarithms, i. e., to volume is added 6.6 board feet, to diameter breast high inside bark is added 8 inches and double bark thickness, and to

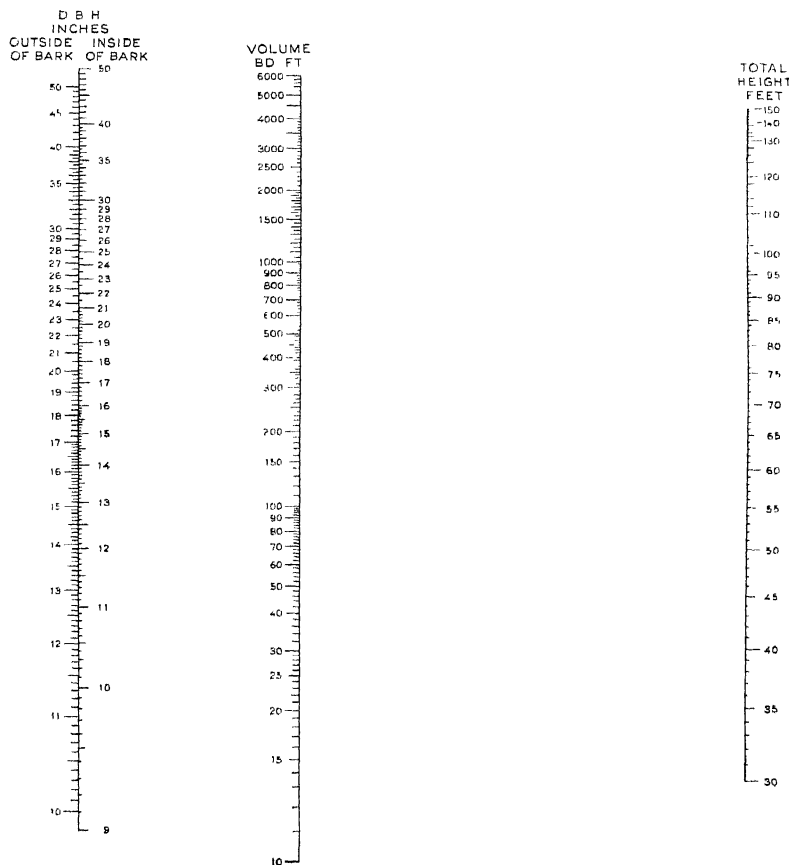


FIGURE 9.—Alinement-chart volume table for mature ponderosa pine (yellow pine).

height is added 4.5 feet. Figures 9 and 10 represent the volume tables in their final alinement-chart form. Tables 3 and 4 give the data in tabular form.

<sup>15</sup> BRUCE, D. and REINEKE, L. H. CORRELATION ALINEMENT CHARTS IN FOREST RESEARCH: A METHOD OF SOLVING PROBLEMS IN CURVILINEAR MULTIPLE CORRELATION U. S. Dept. Agr. Tech. Bull. 210, 88 pp., illus. 1931.

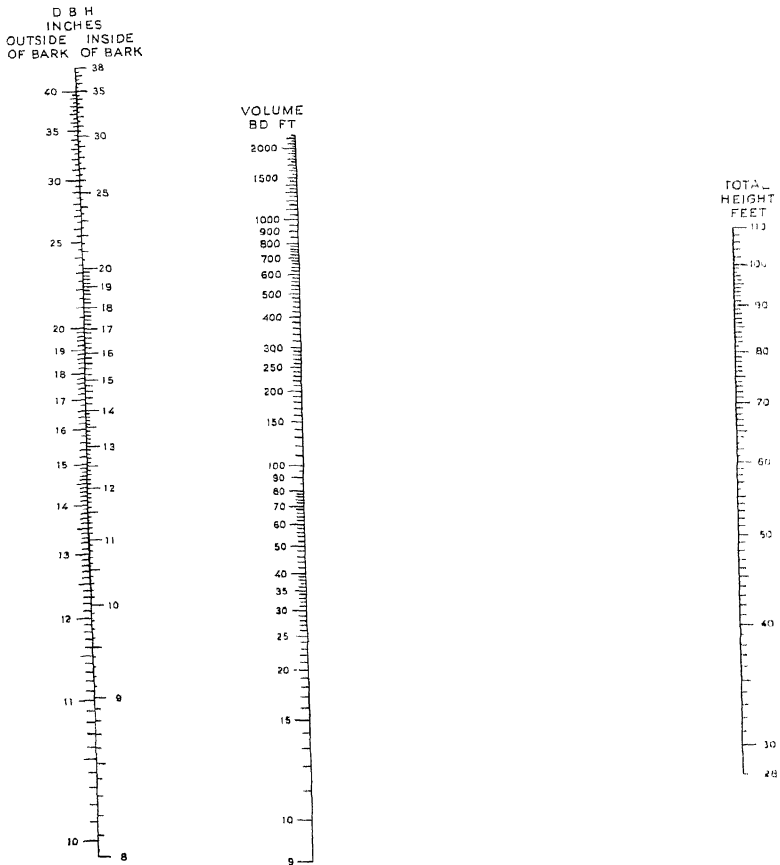


FIGURE 10—Alinement-chart volume table for immature ponderosa pine (blackjacks and intermediates).



TABLE 3.—Board-foot volume table, Scribner rule, for yellow pine (mature ponderosa pine)<sup>1</sup>

Diameter breast high		Volume when the total height of tree is—										Basis (trees)
Outside bark (inches)	Inside bark (inches)	40 feet	50 feet	60 feet	70 feet	80 feet	90 feet	100 feet	110 feet	120 feet		
		Board feet	Board feet	Board feet	Board feet	Board feet	Board feet	Board feet	Board feet	Board feet	Number	
10	9.10	15	18	22	25						0	
11	9.75	22	28	34	40						1	
12	10.50	31	40	50	60	71					0	
13	11.25	41	54	68	82	98					0	
14	12.08	53	70	89	108	128	149				2	
15	12.90	66	88	112	137	163	190	218			2	
16	13.80	80	108	137	169	203	235	270			2	
17	14.70	96	129	166	204	244	284	327	370		5	
18	15.65	113	153	197	240	288	337	387	440	490	7	
19	16.60	130	177	227	279	334	391	448	508	575	5	
20	17.50	147	201	258	317	382	444	514	582	660	9	
21	18.43		225	290	359	430	498	578	656	740	10	
22	19.35		251	322	399	479	560	645	730	825	10	
23	20.25		278	360	440	530	622	717	810	915	15	
24	21.30		306	397	488	590	690	790	895	1,015	10	
25	22.30		339	435	537	645	755	870	984	1,110	13	
26	23.25		369	473	585	707	821	946	1,075	1,210	12	
27	24.15		398	514	635	760	890	1,025	1,164	1,300	14	
28	25.10		430	558	690	825	964	1,110	1,255	1,420	14	
29	26.10			603	740	890	1,040	1,200	1,352	1,525	5	
30	27.10			644	787	950	1,107	1,277	1,445	1,630	11	
31	28.00			688	840	1,016	1,185	1,360	1,540	1,744	7	
32	29.00			727	895	1,080	1,255	1,445	1,640	1,858	5	
33	30.00			770	947	1,145	1,333	1,530	1,740	1,970	1	
34	31.00			820	1,000	1,210	1,409	1,624	1,846	2,100	2	
35	31.95				1,057	1,275	1,480	1,710	1,950	2,210	4	
36	32.90				1,112	1,345	1,558	1,800	2,060	2,320	2	
37	33.90				1,168	1,400	1,640	1,890	2,164	2,425	1	
38	34.80				1,225	1,465	1,720	1,990	2,270	2,520	2	
39	35.75				1,270	1,530	1,790	2,090	2,370	2,650	0	
40	36.80				1,345	1,610	1,880	2,193	2,480	2,790	0	
41	37.80					1,694	1,989	2,300	2,600	2,910	0	
42	38.80					1,760	2,080	2,400	2,710	3,050	1	
43	39.81					1,850	2,180	2,500	2,840	3,200	0	
44	40.90					1,930	2,275	2,600	2,952	3,350	0	
Basis (trees)----		0	3	2	19	63	56	22	9	0	174	

<sup>1</sup> Block indicates extent of basic data. Prepared by the method of least squares. Basis: 174 trees measured by the Southwestern Forest and Range Experiment Station on the Sitgreaves National Forest, Ariz. Malpais area: Average height of mature dominant trees, 95 feet; stump height, 1 foot; top diameter inside bark, 8 inches; scaled in 16-foot log lengths with trimming allowance of 0.3 foot, additional top sections scaled as fractions of 16-foot, 8-inch log. Aggregate deviation, chart 0.43 percent low, average percentage deviation trees 10 inches +, 8.5 percent; values read from alignment chart.

In using these volume tables, discretion is necessary. It should be remembered that they will give results within the standard error specified only if they are applied to trees growing in stands similar to those from which the data were taken. It does not appear, however, that this limitation will greatly restrict the use of the volume tables on stands cut under present Forest Service cutting practice.

TABLE 4.—Board-foot volume table, Scribner rule, for blackjacks and intermediates (immature ponderosa pines)

Diameter breast high		Volume when the total height of tree is—										Basis (trees)
Outside bark (inches)	Inside bark (inches)	30 feet	40 feet	50 feet	60 feet	70 feet	80 feet	90 feet	100 feet	110 feet		
		Board feet	Board feet	Board feet	Board feet	Board feet	Board feet	Board feet	Board feet	Board feet	Number	
10	8.07	10	12	14	15						4	
11	8.97	15	19	23	28						11	
12	9.83		21	28	35	42	49				11	
13	10.75		29	39	49	60	71				21	
14	11.64		37	50	65	80	95	111			10	
15	12.55		46		82	102	122	143			24	
16	13.45		55	64	101	127	152	177			15	
17	14.33			78	122	152	182	213	248		22	
18	15.23			92	144	179	215	252	291		16	
19	16.13			109	167	207	250	292	338		19	
20	17.04			126	190	239	286	335	387	440	496	20
21	18.00				216	272	326	381	441	504	563	10
22	18.85					303	364	428	497	561	633	19
23	19.78			242	270	336	405	476	552	625	702	6
24	20.60			296	370	446	523	609	688	770	847	10
25	21.55			324	408	492	578	667	758	847	938	7
26	22.47			354	442	536	628	724	824	925	1	
27	23.30				482	582	682	785	892	998	3	
28	24.22					627	733	847	955	1,078	1	
29	25.07				560	673	785	910	1,040	1,155	3	
30	26.00					726	850	980	1,116	1,255	3	
31	26.90					775	910	1,055	1,200	1,350	1	
32	27.85					830	970	1,122	1,280	1,448	0	
33	28.75					885	1,035	1,200	1,365	1,540	0	
34	29.60						1,095	1,265	1,450	1,630	0	
35	30.50						1,154	1,349	1,535	1,730	0	
Basis (trees)-----		2	4	31	50	70	50	27	3	0	237	

<sup>1</sup> Block indicates extent of basic data. Prepared by the method of least squares. Basis. 237 trees measured by the Southwestern Forest and Range Experiment Station on the Sitgreaves National Forest, Ariz. Malpais area: Average height of mature dominant trees, 95 feet; stump height, 1 foot; top diameter inside bark, 8 inches; scaled in 16-foot log lengths with trimming allowance of 0.3 foot, additional top sections scaled as fractions of 16-foot, 8-inch log; aggregate deviation, chart 0.12 percent high; average percentage deviation trees 12 inches +, 13.0 percent; values read from alignment chart.

## SUMMARY AND CONCLUSIONS

This paper presents the methods and findings in the development of Scribner board-foot volume tables for cut-over stands of ponderosa pine on the Malpais soil type in Arizona. Improved methods of comparing the volume of the three age classes of ponderosa pine (blackjacks, intermediates, and yellow pines) are described. The methods used, which follow the technique developed by Schumacher and Hall, represent a radical departure from the methods heretofore used in constructing volume tables.

The tables are presented in alinement-chart and tabular form.

For the Scribner board-measure rule and for the data used in this study, the following conclusions may be drawn:

(1) The volume equation for blackjacks does not give a result significantly different from that for intermediates.

(2) The volume equation for yellow pines does give a result significantly different from that for blackjacks or from that for intermediates.

(3) The volume equation for yellow pines gives a result significantly different from that for blackjacks and intermediates combined.

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